Presidential Symposium and Presentation of Top Abstracts

1 Efficacy and Safety of a Single Dose of Exagamglogene Autotemcel for Transfusion-Dependent β-Thalassemia and Severe Sickle Cell Disease

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Elevated fetal hemoglobin (HbF) is associated with improved outcomes in patients (pts) with transfusion-dependent β-thalassemia (TDT) and sickle cell disease (SCD). Exagamglogene autotemcel (exa-cel) is a cell therapy designed to reactivate HbF via non-viral, *ex vivo* CRISPR/Cas9 gene-editing at the erythroid enhancer region of *BCL11A* in autologous CD34+ hematopoietic stem and progenitor cells (HSPCs). We report efficacy and safety data from the first 75 pts dosed with exa-cel in the ongoing CLIMB THAL-111 and CLIMB SCD-121 pivotal trials. Following pharmacokinetic-adjusted busulfan myeloablation and exa-cel infusion, pts (12-35y) are monitored for engraftment, total Hb, HbF, *BCL11A* edited alleles, transfusions, VOCs (SCD only), and adverse events (AEs). Data presented as mean (min-max) unless noted. 44 pts with TDT (age 21.3 [12-35] y) and 31 pts with SCD (22.5 [12-34] y) had been infused with exa-cel at data cut (follow-up 12.3 [1.2-37.2] mo and 9.6 [2.0-32.3] mo, respectively). 26/44 pts with TDT (59.1%) had β^0/β^0 or a β^0/β^0like genotype (β^0/IVSI110, IVS-I-110/IVS-I-110). In 2y period before screening, pts with TDT received 36.0 (15.0-71.0) units RBC/y and pts with SCD had 3.9 (2.0-9.5) severe VOCs/y. After exa-cel infusion, all pts engrafted neutrophils and platelets (median 29 and 43 days in pts with TDT and 27 and 32 days in pts with SCD, respectively). 42/44 pts with TDT stopped RBC transfusions (duration 0.8-36.2 mo); 2 pts had not yet stopped transfusions but had 75% and 89% reductions in transfusion volume. By Month 3, increases in HbF and mean total Hb (>9 g/dL) were achieved, with mean total Hb increasing to >11 g/dL thereafter and maintained. All pts with SCD (n=31) no longer had severe VOCs (duration 2.0-32.3 mo). Mean proportion of HbF was >20% by Month 3, increasing to ~40% at Month 4 and stable thereafter, with mean total Hb >11 g/dL after Month 3. Pts with TDT and SCD with ≥1 y follow-up had stable proportions of edited *BCL11A* alleles in bone marrow $CD34^+$ HSPCs and peripheral blood mononuclear cells. 2 pts with TDT had serious AEs (SAEs) considered related to exa-cel. First pt was previously reported. Second pt had delayed neutrophil engraftment and thrombocytopenia, which were considered related to both exa-cel and busulfan. All SAEs resolved. No pts with SCD had SAEs considered related to exa-cel. There were no deaths, discontinuations, or malignancies. In summary, exa-cel infusion led to elimination of transfusions in almost all pts with TDT and elimination of VOCs in all pts with SCD, with associated clinically meaningful increases in HbF and total Hb that were maintained. Proportions of CRISPR/Cas9-edited *BCL11A* alleles remained stable after >1 y, indicating long-term HSCs were successfully edited. Safety profile was generally consistent with busulfan myeloablation and autologous transplant. These results indicate exa-cel has the potential to be the first CRISPR/Cas9-based therapy to provide a one-time functional cure for TDT and severe SCD.

2 Split Intein-Mediated Protein *trans*-Splicing to Express Large Dystrophins

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Gene replacement therapies mediated by Adeno-Associated Viral (AAV) vectors represent a promising approach for treating genetic diseases. Despite tremendous progress in capsid design and identification of novel serotypes with higher tropism and transduction activity an important constraint on using AAVs is their modest packaging capacity (~4.7 kb). This size restriction can significantly limit their application for many genetic disorders involving large genes, such as Duchenne muscular dystrophy (DMD), which is caused by mutations in the 2.2 MB dystrophin gene that has an 11.2 kb coding region. Affected patients develop body-wide muscle wasting and die from cardio-respiratory failure in their late teens to early 30s. Our group has pioneered the development of miniaturized forms of dystrophin (including micro-dystrophins) that can fit within and be transported by a single AAV vector. Although these smaller dystrophins are surprisingly functional, many clones tested thus far are unstable or showed incomplete rescue of the dystrophic phenotype when tested in DMD animal models and in patients. These observations suggest that the expression of larger dystrophins with additional functional domains is necessary to fully protect from, or reverse, muscle pathophysiology. Here, we present SIMPLI-GT (Split Intein-Mediated Protein Ligation for Gene Therapy), a novel method that allows the expression of large and stable proteins with high specificity and efficiency. This approach exploits the intrinsic ability of split inteins to ligate seamlessly multiple polypeptide fragments into a functional protein *via* a protein *trans*-splicing mechanism. We identified several split intein pairs that can efficiently join two or three fragments and generate, respectively, a large midi-dystrophin or the entire full-length dystrophin. In a proof-of-concept study, we show that

the delivery of two or three AAV vectors results in a strong expression of large and functional dystrophins with a significant improvement of muscle histology and force development. Moreover, using the potent myotropic AAVMYO capsid, we demonstrate that a low dose of 2e13 vg/kg (10-fold lower compared to what is used in clinical studies) is sufficient to express large dystrophins in striated muscles bodywide with an almost complete physiological rescue. Our data show a clear superiority of large dystrophins over miniaturized forms when tested in young or very old dystrophic mice. This novel strategy addresses emerging challenges of AAV-based gene replacement. If successful with DMD, it can be adapted to many other genetic disorders caused by loss-of-function mutations in large genes with a coding sequence beyond AAV packaging capacity.

3 Novel AAV-Capsid-Mediated Delivery of an RNAi Targeting *Atxn2* Extends Survival and Improves Strength and Neuroinflammation in a Mouse Model of Sporadic ALS

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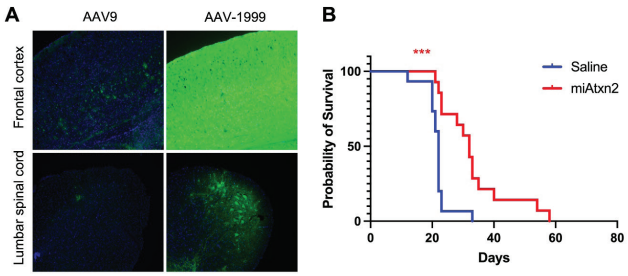
Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by death of motor neurons. A key pathologic feature is the cytoplasmic mislocalization of a nuclear transcription and splice regulator, Tar-DNA binding protein of 43kDa (TDP-43). TDP-43 aggregates in cytoplasmic stress granules (SGs) and leads to toxicity through both cytoplasmic gain- and nuclear loss-of-function. Inhibiting formation of SGs is therefore a promising strategy, and downregulating the SG-associated protein Ataxin-2 (Atxn2) using antisense oligonucleotides (ASOs) prolongs survival by 35% in a mouse model of sporadic ALS (Becker et al. Nature 2017), a strategy that is now in human clinical trials. However, this strategy requires frequent CNS readministration of the Atxn2-targeting ASO and may not effectively reach the brain after intrathecal injections, limiting efficacy and safety. An alternative approach is to provide lasting knockdown throughout the brain and spinal cord after one treatment using AAV-mediated RNAi delivery. If successful, this strategy could be used to treat the vast majority of ALS. We designed miRNAs targeting Atxn2 and tested their efficacy in N2A cells, packaging the most effective candidate into a novel AAV9 capsid variant, AAV1999, engineered in our lab for superior CNS targeting in both mice (**Figure, A**) and nonhuman primates. A dosing study demonstrated 55% knockdown of Atxn2 in the frontal cortex and 25% knockdown throughout the brainstem and cervical and lumbar spinal cord after a one-time intracerebroventricular injection, with GFP-tagging demonstrating selective localization within the spinal cord to anterior horn cells. We then conducted an efficacy study in the same ALS mouse model used in the prior ASO study, in which wildtype human TDP-43 is overexpressed in neurons starting at P7 and mice exhibit a rapid decline in strength, succumbing around P22. After treatment, mean survival was increased by 54% and median survival by 45% ($p<0.002$; **Figure, B**). Mice showed marked improvement across several strength-related measures, including rotarod (2X duration, $p<0.02$); gait (30% improvement, $p<0.001$); kyphosis (66% improvement, $p<0.001$); tremor (34% improvement, $p<0.005$); and foot angling (48% improvement, $p<0.01$), with a trend towards improvement in abdominal droop and limping. Interestingly, mice showed an increase in vertical activity above that seen in wildtype mice, perhaps suggesting an unmasking of an FTD phenotype in the setting of improved strength. Histologically, treated mice showed normalization of the astrogliosis seen in mutant mice to wildtype levels ($p<0.05$). AAV-mediated RNAi targeting Atxn2 is therefore a promising strategy for treatment of the 97% of ALS characterized by TDP-43 pathology.



Clinical Trials Spotlight Symposium

4 Efficacy and Safety at Week 52 and up to Four Years in Adults with Glycogen Storage Disease Type IA (GSDIa): Results from a Phase 1/2 Clinical Trial and Long-Term Follow-Up Study of DTX401, an AAV8-Mediated, Liver-Directed Gene Therapy

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***Background*:** Glycogen storage disease type Ia (GSDIa) results from a deficiency of glucose 6-phosphatase (G6Pase) which is essential for glycogenolysis and gluconeogenesis. DTX401 is an investigational adeno-associated virus serotype 8 (AAV8) vector expressing the human *G6PC* gene. ***Methods*:** An open-label, phase 1/2, dose escalation gene therapy trial (NCT03517085) evaluated the safety and efficacy of a single DTX401 intravenous infusion in 12 adults with GSDIa for 52 weeks. Three patients in Cohort 1 received DTX401 2.0×10^{12} genome copies (GC)/kg, and three patients each in Cohorts 2, 3, and 4 received 6.0×10^{12} GC/kg. Patients in Cohorts 1 through 3 received reactive steroids, and patients in Cohort 4 received a prophylactic steroid regimen to prevent transaminase elevation. All participants entered a long-term follow-up study (NCT03970278) to monitor safety and efficacy for up to 260 weeks after DTX401 administration. The

data cutoff for this analysis was 06-Dec-2022. ***Efficacy Results*:** Mean (SD [range]) total daily cornstarch intake reduction from baseline to Week 52 was 70.0% (23.1 [28-100%]), p<0.0001 among 11 patients with a cornstarch assessment within the ±14 day analysis window for the Week 52 visit. From baseline to last available timepoint in the follow-up study (~4 years for three patients in Cohort 1), mean (SD [range]) total daily cornstarch intake reduction was 65.6% (24.3 [9-100%]), p<0.0001 for all 12 patients. ***Safety Results:*** All patients experienced a treatment-emergent adverse event (TEAE) and a related TEAE; however, no infusion-related or treatment-related serious adverse events (SAEs) were reported. There were three patients with five related TEAEs of hypertriglyceridemia and one patient with two related TEAEs of proteinuria. All SAEs were classified as serious due to hospitalizations and were determined to be unrelated to study drug by both the investigator and study sponsor; all resolved. ***Conclusions*:** DTX401 showed a positive efficacy and safety profile in all treated patients at Week 52 that was sustained for up to four years in patients enrolled in Cohort 1. Patients in all cohorts showed a significant reduction in cornstarch needs from baseline to both Week 52 and to the last available timepoint. All participants remain in the follow-up study and updated efficacy and safety results will be reported.

5 Early Skeletal Outcome after Hematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis Type I Hurler

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Mucopolysaccharidosis type I Hurler (MPSIH) is a rare lysosomal storage disorder caused by defects in *IDUA* gene and characterized by a range of clinical manifestations including severe skeletal dysplasia. While allogeneic hematopoietic stem cell transplantation (allo-HSCT) represents standard of care for MPSIH, skeletal abnormalities progress over time after allo-HSCT, requiring major orthopedic surgery and severely affecting patient's quality of life.We report the early skeletal outcome in 8 MPSIH patients treated with autologous HSPC genetically modified to overexpress human IDUA and followed-up for a median of 3 years after gene therapy (GT). This is a phase I/II HSPC-GT trial (NCT03488394) which enrolled 8 patients (6 M, 2 F; mean age at treatment: 1.9±0.5 years) who lacked a non-heterozygous-HLA-matched cord blood donor and displayed IQ/DQ>70. Primary efficacy endpoint was blood IDUA activity up to supraphysiologic levels at 1-year post-GT. Secondary efficacy endpoints included growth velocity at 1- and 3-year post-treatment. Motor function and spine MRI score at 1- and 3-year post-treatment were defined as exploratory endpoints. Skeletal dysplasia was evaluated in terms of clinical (growth, measures of dorso-lumbar kyphosis and genu valgum by goniometer), functional (motor function by Peabody scale, joint range of motion [ROM]) and radiological (acetabular index [AI] and migration percentage [MP] at hip X-Rays and MRIs, spine MRI score) parameters at baseline and at multiple timepoints, up to 3-year after treatment. All patients are alive at 3-year follow-up and show sustained engraftment of gene-corrected cells with supraphysiologic blood IDUA activity. Urinary GAG excretion reduced to normal or near-normal values by 1-year post GT and remains stable at 3-year follow-up. All patients have progressed along expected growth percentiles of healthy children and exhibit longitudinal growth within the normal range adjusted for age and gender at last follow-up. They also show progressive increase in sitting height and adequate height velocity. Clinical measures of sitting and standing kyphosis showed a trend toward reduction in evaluable patients. Genu valgum is clinically stable with degrees within the physiological range (≤ 10°) in all evaluable patients from year 2 after GT up to the latest follow-up. Motor function showed progressive acquisition of motor skills, while ROM measurement demonstrated improved stiffness at the level of shoulder, elbow, hip and knee joints. Mean AI and MP measured at hip X-Rays and MRIs showed progressive decrease after GT, indicating a reduction in hip dysplasia. Typical spine alterations measured at MRI through a specific score (*Pontesilli et al.*) showed an overall stabilization after GT up to 3-year follow-up (mean score: 3.5 at baseline, 3.3 at 3-year follow-up). HSPC-GT has shown extensive metabolic correction with a favorable safety profile. Early skeletal outcome after HSPC-GT indicates stabilization of skeletal dysplasia at multiple sites up to 3 years post-treatment. A longer follow-up is needed to draw definitive conclusions on the impact of HSPC-GT on MPSIH skeletal manifestations, as compared with standard of care.

Pontesilli S, et al. *J Pediatr*. 2022 Jan;240:297-301.e5.

6 Midbrain Gene Therapy for AADC Deficiency

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OBJECTIVEAromatic L-amino acid decarboxylase (AADC) deficiency is a neurodevelopmental disorder characterized by congenital deficiency of dopamine and serotonin. It presents in infancy with hypotonia, hypokinesia, oculogyric crises (OGC), dystonia, autonomic dysfunction, and global developmental delay. Here we describe interim findings from a Phase 1/2 dose escalation trial to evaluate the tolerability of MR-guided delivery of adeno-associated virus serotype 2 (AAV2)-hAADC to the bilateral midbrain in children and young adults with AADC deficiency.
METHODSTwenty-eight individuals (15F, 13M; median age 8.0 years, range 4-27 years) received AAV2-AADC (dose 4.2×10^{11}-1.5×10^{12} vector genomes(vg)) delivered in a single infusion of up to 300 microliters per hemisphere, targeting the substantia nigra pars compacta and ventral tegmental area. Changes in symptoms and motor function were assessed by caregiver log, neurologic examination and systematic review of home videos for attainment of motor milestones. Changes in dopamine metabolism were assessed by analysis of CSF homovanillic acid (HVA).
RESULTSResults were analyzed for 17 subjects who were followed for at least 12 months (range: 12-45 months). OGC improved in all subjects and resolved completely in 14/17 (82%) after gene delivery. All subjects had severe motor function impairment at baseline, with inability to sit without support. Motor function improvement was observed across the age spectrum. By 12 months post-surgery, head control was attained by 16/17 (94%) of subjects, and independent sitting by 80% (4/5) under age 7 years and 42% (5/12) age 7 years or older at time of surgery. Two subjects (baseline ages 4.8, 4.9 years) walked independently by 36 months. All subjects experienced improvements in mood, sleep, and feeding tolerance. CSF HVA increased from <20% of the lower limit of normal at baseline, to 24-100% at 6-12 months post-gene transfer (median increase 87 nmol/L, range 30-190), consistent with increased brain dopamine synthesis. All subjects tolerated the surgical procedure well. Post-treatment dyskinesia was experienced by all subjects, peaking between 6 and 12 weeks after surgery and improving over 6-12 months.
CONCLUSIONSMidbrain AAV2-AADC gene delivery has now been performed in a total of 35 individuals. The procedure is safe and produces consistent and sustained improvements for up to 5 years in oculogyric crises, mood, sleep, and motor function in patients with AADC deficiency.

7 Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Outcome and Post-Marketing Experience

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Autosomal recessive adenosine-deaminase (ADA) deficiency leads to severe combined immunodeficiency, treatable by enzyme replacement therapy (ERT), allogeneic hematopoietic stem cell transplantation (HSCT) or autologous CD34+ cell gene therapy (GT) following busulfan reduced-intensity conditioning. GT with bone marrow-derived CD34+ cells transduced with γ-retroviral vector (RV) (Strimvelis) was approved in 2016 in the EU. We describe the post-marketing experience of 19 subjects (STRIM cohort) up to 5 years of follow-up and provide extended follow-up data on the 22 subjects treated in the clinical development/named patient program (CDP+NPP cohort, #NCT00598481). We also report on 2 patients who received mobilized peripheral blood (mPB-HE). At data cut-off, all 43 patients were alive, with a median follow-up of 5.5 years (interquartile range (IQR) 3.4-13.0) and intervention-free survival (no need for long-term ERT or rescue HSCT) of 88% (95% CI: 78.7%-98.4%). Most adverse events/reactions were related to disease background, busulfan conditioning or immune-reconstitution. Long-term persistence of

gene-corrected cells, ADA detoxification in RBC, and immune-reconstitution, with 16/17 patients discontinuing IVIg supplementation and responding to most vaccinations, were observed in the CDP+NPP cohort. IFS, transduced cell engraftment, ADA detoxification, immunological reconstitution and the safety profile of the STRIM cohort were in line with the clinical development results. The overall insertion profile of γ-RV was polyclonal in the analyzed samples of the CDP+NPP and STRIM populations. However, a lymphoid T cell leukemia with retroviral insertion near LMO2 emerged 4.7 years post-GT in a patient from the CDP+NPP group, who is currently in clinical remission after 20 months following haploidentical HSCT. In the post-marketing population, we did not find evidence of clonal proliferation, nor new treatment-related events. In summary the safety and efficacy data in the post-marketing population are similar to the clinical development experience and efficacy is persisting long-term. Due to the risk of insertional oncogenesis, long-term safety monitoring remains important (#NCT03478670).

8 GPC3-CAR T Cells Co-Expressing IL15 Mediate Potent Antitumor Activity in Liver Cancer Patients Associated with Toxicity That Can Be Mitigated Using iC9 Safety Switch

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Glypican 3 (GPC3) is specifically expressed in several solid cancers. We optimized a GPC3-CAR with the 41BB costimulatory endodomain and showed that interleukin 15 (IL15) co-expression with this CAR enhances antitumor properties of transduced T cells. We hypothesized that IL15 co-expression (15.CAR) would increase CAR T cell expansion and antitumor activity in children and adults with GPC3+ relapsed or refractory liver cancers. Here, we analyze four phase I studies, two evaluating GPC3-CAR T cells (GAP: NCT02932956; GLYCAR: NCT02905188) and two evaluating the same CAR with IL15 co-expression (AGAR: NCT04377932; CATCH: NCT05103631). Trial objectives included characterizing safety and defining expansion, persistence, and antitumor activity of CAR or 15.CAR T cells. Six-six patients were infused with CAR or 15.CAR T cells at the $3x10^7/m^2$ dose. Toxicity was monitored using the Common Terminology Criteria of Adverse Events v5. *In vivo* persistence was quantified using qPCR and flow cytometry. CAR or 15.CAR T cell antitumor activity was determined by 3D imaging and serum alpha-fetoprotein levels. Compared to the CAR T treated patients, adverse events (AEs) were higher in the 15.CAR group (183 vs. 80, p=0.03), including cytokine release syndrome (one grade 2 event in CAR versus one grade 2 / two grade 4 events in 15.CAR). AEs were more frequent in responders vs. non-responders (15.89 versus 31; p=0.026) and specifically grade 2 AEs were observed more often (2.7 versus 10, p=0.025; **Figure 1A,B**). Expansion of 15.CAR T cells could be rapidly controlled using rimiducid, the inducible caspase 9 (iC9) safety switch alleviating side effects in all three patients that required its use (**Figure 1C,D**).15.CAR T cells had higher peak expansion than CAR T cells in the peripheral blood (6.77×10^7 vs 3.71×10^3 copy number/mcg DNA; p=0.016), and both groups demonstrated similar levels of tumor trafficking based on transgene qPCR from tumor biopsies (**Figure 2A**) which was likely due to the early use of iC9 in the 15.CAR group to alleviate AEs resulting lower number of 15.CAR T cells captured post-iC9 use. Increased expansion in responders versus non-responders was observed in the 15.CAR group (mean 36,17 versus 54,44; p=0.002; **Figure 2B**). In patients treated with CAR T cells, two had progressive disease (PD) and four had stable disease (SD). In patients treated with 15.CAR T cells, three had PD and three had partial responses including resolution of lung metastases. These results correspond to response rates of 0% for CAR T versus 50% for 15.CAR T treated patients (**Figure 2C-E**). Based on results from these phase I studies, co-expression of IL15 leads to increased but manageable toxicity with use of the iC9 safety switch, superior expansion of GPC3-CAR T cells resulting in more potent antitumor activity.

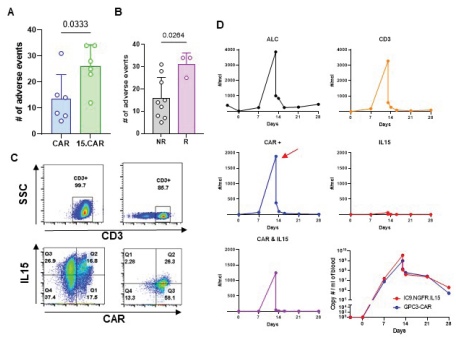

Figure 1. IL15 co-expression increases the frequency of side effects, which can be mitigated with the use of the inducible Caspase 9 (iC9) safety switch: A) Comparison of the number of adverse events (AEs) between patients receiving CAR T cells vs 15.CAR T cells. **B)** Number of AEs between non-responders (NR) vs responders (R) in both CAR T and 15.CAR T groups. **C)** Frequency of CAR+ and IL15+ T cell populations in the peripheral blood of patient "15.CAR 1" before and after rimiducid, the chemical inducer of iC9 dimerization. **D)** Expansion of circulating lymphocyte subsets by FACS and qPCR at indicated time points. Red arrow indicates mitigation of expansion in patient "15.CAR 1" by rimiducid administration. ALC: absolute lymphocyte count.

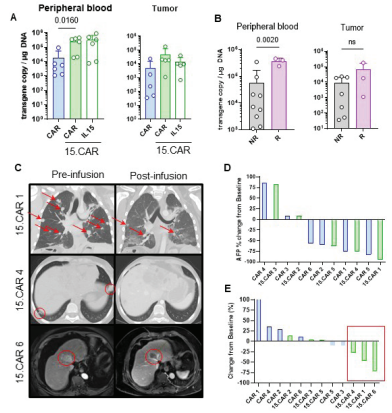

Figure 2. Co-expression of IL15 improves the expansion and antitumor activity of GPC3-CAR T cells in patients. A) Comparison of peak GPC3-CAR T cell expansion in CAR versus 15.CAR T cell-treated patients in peripheral blood and tumor biopsies post-infusion by transgene qPCR. **B)** Peak GPC3-CAR T cell expansion in responders vs non-responders by transgene qPCR. **C)** CT imaging from three responders pre-infusion and 4 weeks post-infusion. **D)** Percent change of serum AFP from baseline (pre-infusion) to post-infusion in all patients with AFP-secreting tumors (n=10). **E)** Percent change of the sum of primary and target lesions pre- and post-infusion for all patients (n=12). Patient "15.CAR 6" treatment response is consistent with complete necrosis on post-infusion biopsy sampling.

9 Danon Disease Phase 1 RP-A501 Results: The First Single-Dose Intravenous (IV) Gene Therapy with Recombinant Adeno-Associated Virus (AAV9:LAMP2B) for a Monogenic Cardiomyopathy

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Background: Danon disease (DD) is a rare X-linked monogenic cardiomyopathy and multisystemic disorder caused by LAMP2 gene mutations resulting in LAMP2 protein deficiency. Male DD patients (pts) develop severe progressive hypertrophic cardiomyopathy (HCM), left ventricular (LV) dysfunction and arrhythmias resulting in mortality at a median age of 19-20 years (y). **Methods:** This open-label, single-dose, phase 1 trial enrolled male DD pts with pathogenic LAMP2 mutations and HCM in two age groups: ≥15 y (n=5) and 8-14 y (n=2). Pts received IV infusion of RP-A501, an adeno-associated virus serotype 9 encoding a normal copy of the human LAMP2B isoform (AAV9.LAMP2B) at 6.7 x 1013 GC/kg (low dose) or 1.1 x 1014 GC/kg (high dose). Transient immunomodulation (IM) included prednisone, rituximab, and sirolimus (for the most recently treated pediatric pts). **Results:** Between June 2019 and March 2022, 7 males (5 adult, 2 pediatric) with DD age 11.7 - 21.1y (median 18.3y) received RP-A501 (N=5 low dose and N=2 high dose). All pts were NYHA Class II at baseline. IM compliance was confirmed in 6/7 pts. All pts are alive and stable at 36 months (m) follow-up. One adult pt with baseline LV systolic dysfunction had progressive heart failure believed related to DD progression and required heart transplant at 5m post RP-A501. Follow-up for the 6 pts with baseline LVEF >40% was 24-36 months (adult pts) and 6-12 months (pediatric pts). All RP-A501- or IM-related adverse events (AEs) were manageable and reversible. A single RP-A501-related SAE of grade 4 thrombotic microangiopathy and renal failure requiring transient dialysis (with full recovery from both) was reported in 1 adult pt in the high dose cohort. No RP-A501 or steroid-related SAEs have been observed to date in the pediatric pts. On baseline endomyocardial biopsy, LAMP2 expression was grade 0 (negative staining by immunohistochemistry) in all patients; 100% (N=6/6) of evaluable pts had cardiac LAMP2B transgene expression within 6m of therapy with reduced LV mass (14-48% decrease from baseline) per echocardiogram. Pts evaluable beyond 6m had stabilized or improved BNP (N=6/6) and troponin (N=6/6) by 6-12m, and stabilized/improved LV wall thickness (N=5/5) and NYHA Class (N=5/5) by 12-18m. These findings persist up to 36m post RP-A501. Improvements/stabilization in BNP diverge markedly from natural history, including those observed in an ongoing prospective study (NHS, UCSD: NCT03766386). In untreated patients of similar age, NPs increased over 3-30m. In this prospective NHS, no patient had improved NYHA class over 6-24 months, in contrast to the NYHA improvements observed following RP-A501. **Conclusions:** Results from this phase 1 trial in DD demonstrate that B- and T-lymphocyte directed immunomodulation enables safe IV RP-A501 gene therapy administration, resulting in cardiomyocyte transduction, LAMP2B expression, improved autophagy, and improved/stabilized serologic, echocardiographic and clinical parameters associated with prognosis in HCM. These data support activation of a phase 2 clinical trial.

10 Clinical Dose-Response Relationship and Safety Profile of rAAVrh8-HexA/HexB

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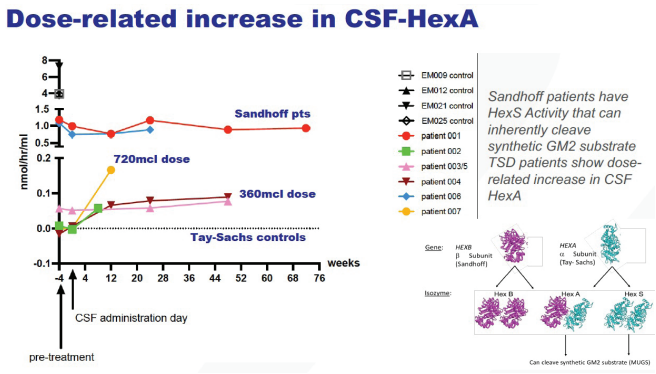
Tay-Sachs Disease (TSD, HEXA mutation) and Sandhoff disease (SD, HEXB mutation) result in lysosomal storage of GM2 gangliosides with severe neurodegenerative consequences. Infantile TSD and SD are fatal within the first several years. Juvenile-onset patients regress after age 2 and also have poor outcomes. Previously, a two-vector rAAVrh8 gene therapy (rAAVrh8-HexA/HexB) showed bioactivity and partial phenotypic correction in animal models and in a trial in 2 SD infants. At the time of presentation, 7 patients (4 infantile and 3 juvenile) will have been treated with bilateral intrathalamic delivery (BiTh) to exploit axonal transport, along with intracisternal/intrathecal (CSF) injection. Patients were treated with rituximab, corticosteroids and sirolimus, the latter two for 3- and 6 months post. Three dose levels were tested: a starting BiTh dose (STD) of 180 mcl (5.87E+12vg BiTh; 1.42E+14vg total), a low-dose (LD) of 360 mcl (1.17+E13vg BiTh; 1.95E+14vg total), and a mid-dose (MD) of 720 mcl (2.35E+13vg BiTh; 2.18E+14 total). Among infants, one each will have been treated at the STD, LD and two at the MD levels, while among juveniles, 2 were LD and 1 was MD. Follow-up ranges from 2 to 24 months. Outcomes included safety, CSF biochemistry, MRI/MRS/DTI and neurodevelopmental observations. CSF HexA enzyme levels showed a consistent dose-related increase in TSD patients after gene therapy [Figure], while CSF GM2 decreased. Study procedures were well tolerated. Injection-related MRI changes partially resolved on later scans. Infants showed partial stabilization, including preservation of oral feeding, and improvement of myelination in the striatum and parietal lobes. Serious adverse events (SAEs) included infections (including one fatal C. diff infection at 5.5 months post-therapy) and seizures (disease-related). In juveniles, dystonic posturing worsened after dosing in all 3 patients treated. The demonstration of a clear dose-response in CSF HexA levels confirms the feasibility of combined BiTh and CSF injection for GM2 gene delivery.



AAV Capsid Biology

11 Identification of Host Factors Critical for rAAV2.5T Transduction of Polarized Human Airway Epithelia

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rAAV2.5T was selected from the directed evolution of an AAV capsid library in human airway epithelium (HAE) cultured at an air-liquid interface (ALI). The capsid gene of rAAV2.5T is a chimera of the N-terminal unique region of AAV2 VP1 (VP1u) and the VP2 and VP3 regions of AAV5 with a single A581T mutation. We conducted two rounds of genome-wide CRISPR screens of rAAV2.5T transduction in HeLa-S3 cells using the Brunello gRNA library. This study identified that KIAA0319L (AAVR), GPR108, and WRD63 are critical host factors for AAV2.5T transduction in HeLa cells. We next investigated the role of these host factors in rAAV2.5T transduction of the polarized HAE-ALI cultures, in which the genes encoding these host factors were knocked out individually. AAVR was identified as the proteaceous receptor of various AAVs to infect cells, including AAV2 and AAV5. However, we found that although AAVR was essential for rAAV2.5T transduction in polarized HAE-ALI cultures, the apical entry of rAAV2.5T was independent on AAVR, suggesting that another proteaceous receptor may also be used to mediate rAAV2.5T entry of HAE-ALI. GPR108, a G protein-coupled receptor, is known as an essential host factor for transduction of rAAV2, but not for rAAV5. On the other hand, we observed that GPR108 knockout aborted the rAAV2.5T transduction in HAE-ALI; however, the application of doxorubicin (DOX) reinstalled the transduction of rAAV2.5T in the GPR108-KO HAE-ALI cultures. DOX is an FDA approved chemotherapy agent for cancer treatment. It is a DNA topoisomerase II inhibitor and also demonstrates inhibition of proteasome activity. Application of DOX increased rAAV2.5T transduction in HAE-ALI cultures by >100-fold. Mechanistically, DOX enhanced rAAV2.5T transduction was primarily mediated by altering the intracellular trafficking pathway of the vectors, but not the inhibition of proteasome-dependent degradation. Importantly, we identified that a sequence of 14 amino acids at AAV2 VP1u determined the rAAV2.5T dependence of GPR108 for transduction. We further confirmed that AAV2 VP1u specifically interacted with the cytosolic portion of GPR108, which likely facilitates rAAV to be released from trans-Golgi network (TGN), and the use of DOX assists rAAV to escape the TGN and enter the nucleus while GPR108 is absent. In summary, rAAV2.5T utilizes AAVR and GPR108 for productive transduction in HAE-ALI, but it does not solely use AAVR for vector entry. DOX facilitates rAAV2.5T to escape the TGN through a GPR108 independent pathway for nuclear import.

12 AAV2's Defective Trafficking in the Absence of the AAV-Receptor is Due to a Block in Endosomal Escape

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Seven years have elapsed since the paradigm-shifting discovery of KIAA0319L, an essential receptor for adeno-associated virus infection, a protein since then named AAVR. However, the understanding of the mechanistic function of this protein in the transduction pathway of AAV has lagged. Viral overlay assays and resolved cryo-electron microscopy structures of the AAV2-AAVR complex performed by independent laboratories suggest that AAV2 physically interacts with AAVR. Multiple labs have also confirmed a complete lack of functional transduction - understood as transgene transcription and translation - in the absence of AAVR. This suggests the presence of a block in the transduction pathway of AAV2. In this work, we aimed to reveal the nature of such block, in the context of AAV2 transduction, in the absence of AAVR. Our study, performed in HuH-7 naïve and in-house generated HuH-7 AAVR-KO cells, suggests that AAV2 vectors can still physically enter cells lacking AAVR, as demonstrated by the presence of intracellular vector genomes and A20 antibody signal, which recognizes intact AAV2 capsids. However, in the case of AAVR-KO cells, this vector uptake led neither to RNA (as shown by RNA-FISH) nor to, understandably, protein expression. In contrast, transgene translation and expression were readily detectable in naïve HuH-7 cells already 24h post-transduction. To understand whether the block in transduction occurred upstream or downstream of the nuclear entry, we used transmission electron microscopy (TEM) to detect rAAV2 particles in naïve and AAVR-KO HuH-7 cells. To our surprise, we observed clear signs of nuclear envelope breakdown in naïve cells, which is consistent with the mechanism of nuclear entry described for other parvoviruses. We observed no such signs in AAVR-KO cells, suggesting the pathway block in AAVR-KO cells was upstream of nuclear entry. Realizing that the AAV2 phenotype in AAVR-KO cells mimics the well-studied phenotype of AAV2 virions lacking the VP1 capsid protein, we next investigated whether a defective endosomal escape could be a driver behind the observed block of AAV2 transduction in AAVR-KO cells. We could detect A20 signal (intact capsids) in both naïve and AAVR-KO cells, but when studying the VP1-unique (VP1u) exposure in transduced cells, we could only detect A1 signal, which recognizes the VP1u region, in naïve HuH-7 cells. The currently accepted theory is that endosomal acidification leads to the exposure of the PLA_2 domain present in the VP1u region that is required for endosomal escape. Thus, our data suggest that either AAVR regulates such acidification or that acidification *per se* is

not sufficient, intracellularly, to mediate the conformational change in the AAV capsid. To study this, we used pH dependant fluorescent lifetime imagining microscopy (pHLIM) to quantify the sub-cellular pH of the individual endosomal vesicles accurately. Preliminary data in naïve and AAVR-KO showed no major differences in the pH in both cell lines. Hence, this work challenges the commonly accepted theory that endosomal acidification is the only required step for VP1u exposure and opens the possibility for the AAVR-AAV2 interaction to be essential for such a process. The absence of this hypothesized interaction in HuH-7 AAVR-KO cells would trap the vectors in the endosomal vesicles, which would be consistent with the observed lack of functional transduction AAV2 in this knock-out line.

13 Modifying Immune Responses to Adeno-Associated Virus (AAV) Vectors by Capsid Engineering

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Adeno-Associated Virus (AAV) vector-based gene therapy may be impaired by *de novo* immune responses towards the AAV capsids or encoded transgene products. With the aim of reducing potential innate immune responses in cells infected with AAV, we generated a capsid-engineered AAV vector displaying an immune modulatory peptide on its capsid that is known to interfere with innate immune signaling. Insertion of this peptide did neither affect capsid assembly nor production efficiency. The new capsid variant, termed AAV2.MB, outperformed AAV2 in terms of transduction efficacy in human monocyte-derived dendritic cells (moDCs) and in primary human hepatocyte (PHH) cultures. In line with our hypothesis, AAV2.MB and AAV2 differed in triggering innate immune response activation in human primary cells. Specifically, induction of the expression of type I interferons was lower in moDCs treated with AAV2.MB. Importantly, AAV2.MB vector administration either intravenously or intramuscularly resulted in a markedly reduced $CD8^+$ T cell response against the AAV vector-encoded EGFP transgene product. Beyond that, humoral responses against AAV capsids measured by anti-AAV2 IgG2a antibodies were mitigated and delayed in AAV2.MB-injected mice independent of the route of administration. To conclude, by incorporating an immune-modulatory peptide into the AAV2 capsid we could modify the activation of innate as well as adaptive immunity in response to AAV2 vectors.

14 Structural and Kinetic Characterization of Anti-AAV9 Monoclonal Antibodies Derived from Patients Post-Zolgensma Treatment

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The utilization of adeno-associated virus (AAV) as a gene transfer vehicle has become increasingly feasible as a clinical option in recent years. Following the FDA approval of Zolgensma®, an AAV9 based biologic to treat children under the age of two with spinal muscular atrophy (SMA), there is a growing interest in expanding AAV9 usage due to its ability to transduce cardiac and skeletal muscle, liver, pancreas, eye, and its capability to cross the blood-brain-barrier to transduce the central nervous system (CNS). However, the presence of pre-existing neutralizing antibodies (NAbs) against AAV9 capsids in a large percentage of the population could reduce the efficacy of AAV9 gene therapy and may lead to the exclusion of patients from treatment. A strategy to circumvent the immune response of a patient for AAV-mediated therapeutic gene delivery is the development of engineered vector capsids by either directed evolution or rational design that are then able to escape antibody recognition. In order to pursue this strategy, the interactions of the NAbs to the capsid binding need to be characterized. Previously, our lab as well as others have generated mouse monoclonal antibodies targeting the AAV9 capsids to simulate the immune response against the capsid and map the major antigenic regions. However, this approach has faced criticism as mouse-derived antibodies may not fully mimic the behavior of human-derived antibodies. Here, we present the structural and kinetic characterization of human monoclonal antibodies that were obtained from patients that received Zolgensma®. Specifically, we have determined the binding sites of 21 antibodies obtained from three patients to the AAV9 capsid by cryo-electron microscopy and three-dimensional image reconstruction. Our data shows that the 2-fold capsid surface is the antigenically dominant region as approximately three quarters of the antibodies bind there. The binding interactions and kinetics for these antibodies were analyzed using biolayer interferometry and compared to the previously developed murine antibodies. All the antibodies neutralize AAV9 transduction, and some also cross-react and neutralize a range of different AAV serotypes. Additionally, there is a strong correlation between Fab binding affinity and the IC50 values for the corresponding mAb. Structural and kinetic comparisons of human and murine antibodies will be presented along with antibody escape variants.

15 Co-Identification and Characterization of Host and Viral Protein Interactomes during AAV Transduction by Two Different Proximity Labeling Methods

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Recombinant adeno-associated viral vectors (rAAV) are considered promising vehicles for gene therapy. However, high dose of AAV administration and insufficient transduction efficiencies still pose difficulties on therapeutic applications. Efficient transduction is determined by the life cycle of AAV and more significantly the molecular interactions between the viral vectors and the host proteins. A better understanding of the cellular proteins involved in AAV transduction throughout the transduction pathway of AAV will lead to improved efficiency of AAV-derived vectors. The proximity-dependent biotin identification method allows detecting protein-protein interactions with a promiscuous biotin ligase fused to a bait protein. These biotinylated proteins can be selectively isolated by biotin-streptavidin capture and identified by mass spectrometry. We grafted two types of biotin ligase, BioID2 or TurboID onto the surface of AAV2 capsid. BioID2 is a smaller biotin ligase, and the major disadvantage is its slow kinetics, which necessitates labeling with biotin for 18-24 hours. By contrast, TurboID is greater in size but enable the labeling in 10 minutes. Both engineered AAV2 capsids can biotinylate un-biotinylated substrates just like a purified biotin ligase. During AAV transduction in HEK 293 cell, biotin and ATP were added to the culture medium. Twenty-four hours post infection, proteins were then selectively isolated by streptavidin-coated beads for mass spectrometry analysis. Finally, we found four host proteins which showed increased AAV2 transduction in HEK 293 cells and HeLa cells after knock-down by siRNA treatment. Furthermore, specific combinations of two host siRNAs showed higher transduction efficiency in AAV2-infected HeLa cells, compared to a single siRNA treatment. This was also observed in multiple serotypes including AAV1, AAV3b, AAV5, and AAV9. Those four host proteins are not involved in the attachment of AAV to the cells, internalization, nuclear import, and genome release (uncoating). And yet, knock-down of the host proteins significantly increased luciferase transcript through recruiting transcriptional machinery onto the AAV vector genome. Our findings will advance our understanding of the molecular interaction between the host proteins and AAV during transduction, and consequently contribute to improving the efficacy of AAV transduction.* G.G., and J.X are co-corresponding authors

16 Multiplexed Characterization of AAV Cellular Tropism and Promoter Expression in Large Animals Using Single-Cell RNA Sequencing

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In order to develop the best AAV vector for gene delivery, it is important to identify a serotype that is specific to a cell-type of interest and engineer the payload so that it is capable of high transgene expression in relevant disease contexts. Existing AAV serotype screening methods based on AAV genome detection do not provide information on vector functionality nor levels of transgene expression. Other methods relying on bulk next-generation sequencing of barcoded genomes do not confer cell-type resolution within a target tissue. We have developed a platform using expressed barcodes combined with single-cell sequencing that allows for high-throughput multiplexed screening of functional AAV expression across multiple cell types within different organs of relevant animal models including mouse, horse, pig, and non-human primates. Using this screening platform, we are able to identify an optimal cell-type specific AAV serotype and run head-to-head comparisons of regulatory elements to identify promoters for high expression. As an example of this platform, we identified AAV serotypes capable of transducing chondrocytes and synovial cells for the treatment of osteoarthritis (OA). 13 serotypes, each at a dose of 1E12 vg, were pooled and injected into osteoarthritic horse joints (n = 4 joints). 4 weeks after injection, the joint tissue was assessed for GFP expression and processed. Positively transduced chondrocytes and synovial cells were run through the 10x Genomics single-cell system. Following next-generation sequencing, we processed our data to match cell-types with functional internalized AAV genome barcodes. We identified several serotypes with up to 32.8x higher transduction in chondrocytes as compared to AAV2.5, a serotype currently used in clinical trials of OA. Follow-up injection of our chondrocyte-specific and synovial-specific serotypes at doses ranging from 1E12 to 8E12 vg into non-diseased and OA joints (n = 11 joints) phenotypically confirmed the the results of our screen and resulted in transduction percentages in a range of 1 - 5% and 0.5 - 15%, respectively. We used the same screening approach to optimize regulatory elements for a gene of interest for the highest relative number of transduced cells and level of expression across different cell-types within multiple tissues. A pooled library of 47 combinations of enhancers, promoters, introns, and poly-A signals was tested in mouse lungs (n = 10) and livers (n = 9). From the resulting data, we were able to identify a ubiquitous enhancer-promoter-intron combination with active transcripts and expression detectable in twice as many cells as a ubiquitous CMV promoter. Continuing the expansion of our platform's capabilities, the above methods have been combined to identify serotypes and promoters for a heart failure pig model. The top 3 serotypes (from 17 serotypes) and the top 3 promoters (from 47 combinations) were identified and validated

in a mouse model transcriptomically and histologically. These vectors along with single-stranded and self-complementary variants were injected in a heart failure pig model (n = 3 pigs) to achieve successful gene expression. Taken together, this work shows the broad-ranging ability of our platform to produce optimal AAV vectors for gene delivery. Through the combination of single-cell RNA sequencing with transcriptomic readouts of barcoded libraries, we are able to identify cell-type specific serotypes and highly expressing regulatory elements in relevant animal models for disease.

17 A New Class of AAVR Binders: Structure of a Goat Adeno-Associated Virus (AAVGo.1) Complexed with the Human Adeno-Associated Virus (AAVR) Receptor

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Adeno-associated viruses (AAVs) are compact (~250 Å diameter) non-pathogenic viruses that are useful for gene therapy. Viral capsid structure modulates cellular entry. AAV is first anchored to cells using glycan attachment factors, then cell entry is mediated by the Adenovirus Associated Virus Receptor (AAVR) and several potential co receptors. Most AAV serotypes, including AAV2, bind AAVR using the second of five AAVR polycystic kidney disease (PKD) repeat domains (PKD2) whereas the distantly related serotype AAV5 binds PKD1. The two modes of binding (PKD2 vs PKD1) have been visualized using cryo-EM: PKD2 binds to a capsid area distinct from the PKD1-binding region. In order to examine the full potential diversity in AAVR-capsid interactions an investigation of the goat parvovirus, AAVGo.1, was initiated. Goats are the most distant relatives of human hosts for which there is known cross-species AAV susceptibility. Virus like particles of AAVGo.1 were produced using Sf9 cells and purified using cesium chloride gradient ultracentrifugation. Various AAVR domains were expressed in E. coli and purified using FPLC. ELISA assays were used to detect receptor binding and also for neutralizing antibody binding comparisons between AAV5 and AAVGo.1. Virus receptor complexes were then visualized using cryo-EM. Cryo-EM structures of both AAVGo.1 (2.9 Å resolution) and AAVGo.1 bound to human AAVR (2.4 Å resolution) were solved. AAVGo.1 is most similar to AAV5 and also binds to PKD1 but with a rotation of ~1 Å compared to PKD1 bound to AAV5. Therefore, AAVGo.1 becomes the second member (alongside AAV5) of a distinct class of AAV in terms of their mode of AAVR binding (to the PKD1 domain). The majority of virus-receptor interactions are conserved within each class and this has important implications for vector engineering.

AAV Vectors - Product Development Manufacturing: Downstream

18 Exploring the Robustness and Throughput of Size Exclusion Chromatography of Recombinant AAVs

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Adenoassociated virus (AAV) gene therapies have advanced over the past decade to become a promising class of therapeutics capable of treating intractable diseases. Establishing an AAV as a well characterized drug product is however a major undertaking because they are inherently heterogeneous, containing full, partially filled, and empty capsids as well as aggregates, fragments and process-related impurities. As with the antibody pipeline, liquid chromatography (LC) assays have emerged to provide CQA measurements and to aid drug product engineering, process development, formulation, and release testing. With that, there is a need to continue exploring the capabilities of these new LC assays, learn their limits and make new insights about their robustness. Size exclusion chromatography (SEC) is one of the most important techniques being adopted. It is favored not only for its size variant profiling but because it can be readily coupled with multiple wave-length UV, fluorescence (FL), multiangle light scattering (MALS) and refractive index (RI) detectors so that multiple physicochemical measurements can be extracted from a single run. With current practices, analytical SEC for AAV analysis makes use of ≥ 5 µm diameter 300 to 1000 Å pore size packing materials and large volume columns made from stainless steel hardware. This means that analyses can be time consuming, use significant amounts of sample and be plagued by non-specific interactions that can impair the quantitation of important impurities. As a result, we are exploring separations of AAV2, AAV5 and AAV9 using the above starting points and systematically demonstrating the effects of using high efficiency 2.5 micron packing materials and low adsorption column hardware. Enthalpy contributions and solute retention will be reported along with an investigation into the potential for shear force effects to impact critical measurements. Moreover, a new simple experimental design will be employed for a method robustness check using an approach that requires only 8 quick experiments. It is hoped that these SEC studies will provide new insights on robustness and throughput considerations that the field can more broadly apply in support of the burgeoning AAV pipeline.

19 Novel Repression System for Gene Expression Regulation during Recombinant Adeno-Assoiciated Virus and Lentiviral Vector Manufacturing

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Ubiquitous promoters are widely used for gene expression by viral vectors. However, the same gene also expresses in vector packaging cells. Gene of interest (GOI) could be toxic or disruptive to viral vector

packaging, resulting in either low yield or poor quality of vectors during manufacturing. There were several potential solutions to this challenge. Tet-on inducible or tissue-specific promoters could be used, yet the application is restrictive to meet different needs. There were studies using RNAi-based technology to silence GOI expression. However, this system may not be widely applicable to all GOI expressions. Some research used the TRiP system for exogenous protein translation inhibition, but it has no control over RNA encoding genes. In this study, we developed a gene expression regulation system, which overcomes the defects mentioned above. The CuO-Cymr repression system includes a short DNA element, which could insert into a ubiquitous promoter. The element responses to a repressor which is constructed into the manufacturing cell line. The induction results in significant gene silencing. The leaky expression is minimal compared with other inducible systems. Importantly, it is applicable to different GOI and regulates the expression of both protein and RNA. We evaluated the system in toxic gene packaging by recombinant adeno-associated virus or lentiviral vectors. Both vectors achieved high yield by packaging toxic genes, otherwise, vectors could be barely produced. The inducible system has no changes over gene expression or function for downstream applications of the viral vectors.

20 Ahead of the Game: A Scalable Purification AEX Platform Capable of Achieving <10% Empty Capsids

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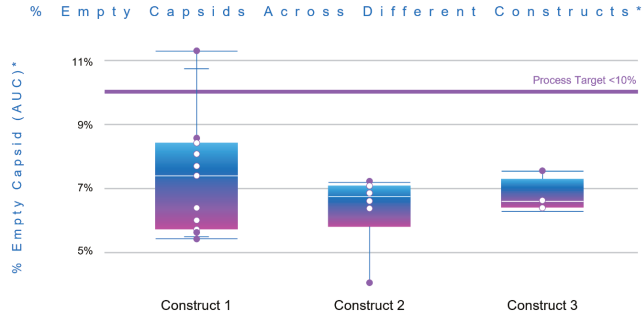
Control of empty capsids is a significant challenge for the manufacture of AAV therapeutics. The elevated presence of empty capsids in AAV Drug Product may cause undesirable safety issues and the level must be controlled to ensure patient safety. The purification process for AAV can be designed to remove empty capsids to limit exposure to patients. The traditional approach has been to use gradient ultracentrifugation. Gradient ultracentrifugation can separate empty from full capsids by exploiting their relative differences in density and can work well in small scale setting, but can be challenging to implement and control in a manufacturing setting. Furthermore, as AAV bioreactor titers increase, ultracentrifugation becomes more challenging to effectively scale up. To meet the growing clinical and commercial demands of AAV, alternative approaches that can scale up and deliver comparable levels of empty capsids need to be developed. Many organizations and manufacturers have adopted anion exchange chromatography as the preferred process of choice to ultracentrifugation. However, a limited number of these methods can deliver the same level of empty capsids as ultracentrifugation which leave therapeutic developers with having to choose between yield and product quality. Our presentation will introduce an Anion Exchange chromatography step (AEX) that delivers on both yield and product quality, by achieving <10% empty capsids, a high yield of 67-88% vector genomes, and is scalable to 2000L. The AEX step consistently produces <10% empty capsids across multiple constructs with the same capsid. It also delivers the same level of purity across other major serotypes including, but not limited to AAV1, AAV2, AAV6, AAV8 and AAV9. Reproducible operational success at the 50L, 500L, and 2000L scales (N=23) with comparable product quality and yield has been possible due to the isocratic nature of the step. This makes the platform manufacturing friendly, automatable, and very robust.



In our talk, we will describe how the AEX platform was developed, implemented for multiple serotypes and constructs, scaled-up and compared to ultracentrifugation. The plug and play applicability and workflow will also be reviewed. Furthermore, we will discuss the scalability of this step and how it helps to streamline manufacturing operations, reduce Cost of Goods (CoGs) and enable straightforward late-stage process characterization and commercial manufacturing. Finally, we will outline the value proposition offered by the AEX platform; a robust, efficient manufacturing step that can be executed at any scale and consistently delivers high product quality. The use of this AEX step can lead to higher productivity manufacturing and enable therapeutic developers to treat indications with larger patient populations in a safe and economical manner.

21 Impact of Downstream Buffer pH and Ionic Strength on rAAV Capsid Stability

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A production process consisting of suspension cell culture and chromatographic purification is becoming increasingly popular for large-scale manufacturing of rAAV vectors for gene therapy. Before the preparation of the rAAV drug product in the formulation buffer, rAAV vectors face a series of downstream purification buffers across different unit operations like lysis, filtration, chromatography, and tangential flow filtration. The downstream processing of rAAV vectors can take 3- 7 days. During this time, the rAAVs are exposed to environments of different pH ranging from 9.0 to 3.0. They may also be held in a particular environment for over 24 hours to accommodate the processing timeline. Moreover, downstream processing buffers also span an extensive range of ionic strength (10 mM -500 mM) and may include additives such as metal ions and surfactants. Exposure to unfavorable ionic strength and pH buffers can lead to various degradation issues. In particular, chromatography operations require low ionic strength buffers and harsh pH conditions. In this work, we systematically evaluated the stability of AAV capsid in predefined buffer components ranging from pH 3 to 9 and ionic strengths of 50 to 300 mM, along with commonly used purification additives. The rAAVs were incubated in various buffers for 0, 2, 6, 12, and 24 hours. They were then analyzed on analytical anion exchange (AEX), hydrophilic

interaction liquid (HILIC), and size chromatography exclusion (SEC) chromatography to monitor the changes in the product profile. The AEX result indicated lower ionic strength impacts AAV capsid stability more significantly than pH. Furthermore, empty and full capsids showed different stability profiles. Buffers with 50-120 mM ionic strength significantly changed the AEX peak profile, particularly in the mid-pH range. Given that the above pH and ionic strength ranges are most commonly utilized during chromatographic operation, assessing their stability and solubility is critical. Based on our study, we recommend strategies to limit the exposure of AAVs to unfavorable buffer conditions as needed, such as the implementation of inline dilution during product loading onto AEX and the adjustment of pH and IS after chromatographic elution.

22 Challenging the Bind and Elute Convention: A Novel Approach to Partitioning Method for Full rAAV Capsid Enrichment Using AEX Chromatography

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Two of the largest hurdles in the downstream purification of recombinant Adeno-Associated Virus (rAAV), at present, are variable distribution of empty and full capsids produced in each bioreactor batch and that empty capsids co-purify with genome containing (full) rAAV vectors during chromatographic separation. Several publications have detailed methods of separating empty and full recombinant rAAV particles by strong anion exchange chromatography (AEX) (Dickerson et al., J.F. Wright et al.). To date, these reported methods have relied upon a bind and elute strategy to achieve the high degree of chromatographic resolution required to separate empty and full capsids. These gradient-based elution methods typically are very sensitive to variability in load composition and mobile phase composition and require a tightly controlled fractionation strategy, all of which pose a challenge to robust scale-up. This presentation will detail a novel method of enriching full genome containing rAAV over AEX that addresses the challenges of process robustness and scalability. Modulating the mobile phase composition during the AEX load enables the flowthrough or "partitioning" of empty capsids while selectively retaining full rAAV vectors. Minimizing empty capsid binding simplifies the elution strategy, enabling better process control, which can be a significant benefit during tech transfer and scale up. Furthermore, the partitioning technique is robust to changes in starting material (serotype, transgene, vector titer, and empty/full distribution in upstream samples). Using this method, we have demonstrated full capsid enrichment at levels comparable to those obtained by the standard industry practice of density gradient ultracentrifugation across a range of scales and multiple serotypes including 2, 2i8, 8, 9.

23 Preserving High Transduction Efficiency - Serotype Agnostic Purification of Adeno-Associated Virus Using Adsorbents Functionalized with In Silco Discovered Peptide Ligand

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Preserving the transduction efficiency of adeno-associated virus (AAV) is crucial in gene therapy and gene editing applications. High transduction efficiency leads to greater therapeutic efficacy and better patient outcomes. On the other hand, low transduction efficiency can lead to insufficient therapeutic gene expression and, therefore, ineffective treatment. Additionally, preserving transduction efficiency is also important in optimizing the safety of AAV-mediated gene therapy, as it reduces the amount of virus needed to be administered, minimizing potential adverse effects. Adeno-associated virus receptor (AAVR) is an important factor in the transduction efficiency of AAV. AAVR is a cell surface protein that is required for AAV to enter target cells and deliver its genetic payload. By identifying the specific cell types and tissues that express AAVR, researchers can optimize the targeting of AAV to the desired cells and increase the efficiency of transduction. Furthermore, understanding the regulation of AAVR expression and its interaction with other cellular components can lead to the development of novel strategies for enhancing AAV transduction efficiency. Additionally, the A20 antibody is an important component in the use of AAVs for gene therapy. AAVs are commonly used as vectors for delivering therapeutic genes to treat various diseases, but their use can be limited by the immune response generated against the virus. The A20 antibody specifically targets the A20 protein, which is known to play a role in regulating the immune response. By blocking the activity of the A20 protein, the A20 antibody can enhance the transduction efficiency of AAVs, thereby increasing the effectiveness of gene therapy. In this study, we have tackled the issue of AAV stability by proposing a methodology to purify AAV Serotype 2 (AAV2) from Human embryonic kidney (HEK293) cell lysate using peptide-based adsorbents, with mild elution conditions that will prevent capsid structure alteration, which is typically caused by reduction of pH and hence preserving the transduction efficiency. Docking studies were performed with the VP1 sequence of AAV2 against the sequences of AAVR and A20 antibodies. Eight different peptides were discovered via Insilco modeling. These were then synthesized on the Toyopearl NH2-750F resin bead. Further, binding efficiency, selectivity, and recovery in the purification of AAV2 using the Insilco-discovered peptides were evaluated. 20 mM Bis-Tris, 25 mM NaCl buffer at pH 7.0 maximizes the capture of AAV2 on the peptide-based adsorbents. A 10 ml injection of HEK293 clarified lysate on a 0.5 ml column yielded AAV recoveries of up to 70% with great selectivity, using mild elution conditions such as a buffer containing 1.0 M Magnesium chloride at pH 6.0. These recoveries were hugely comparable to the commercially available resins, where the capsids are eluted in a low pH (2.0 - 2.2) environment. HEK293 host cell protein (HCP) logarithmic removal value (LRV) of 2.2 - 2.8 was achieved by the peptide-based resins compared to an LRV of 2.8 obtained from the AAVX and AVB Sepharose HP resins. Bind and elute studies on pure AAV1, AAV5, AAV6, AAV8, AAV9, and AAVrh10 samples also indicated the ability

of some peptide-based resins to capture these serotypes effectively. Collectively, these results demonstrate the potential for an alternate selective chromatographic process for purifying AAV2 and potentially other AAV serotypes from HEK293 clarified cell lysate in a neutral pH environment, thus preserving the transduction efficiency.

24 IsoTag AAV - An Innovative, Scalable, Non-Chromatographic Method for Streamlined AAV Manufacturing

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Purification of adeno-associated viruses (AAVs) is a rate-limiting step of therapeutic development which impacts the cost, safety, and efficacy of the final product. Traditional separation processes - filtration accompanied by affinity capture - are the industry standard for primary contamination clearance but are inefficient, costly, and impractical when applied to gene therapy viral vectors. Isolere Bio, Inc. has developed IsoTag-AAV, a purification process that combines filtration and affinity separation into a single processing step via the novel mechanism of liquid-liquid phase transition. Briefly, AAV capsids can be sequestered and concentrated into IsoTag-AAV droplets away from contaminants and then subsequently released from the droplets in a user-triggered manner for collection of pure product. The entire process can be performed with a standard tangential-flow filtration (TFF) machine and commercially available using hollow fibers between 0.22 and 0.65-micron pores. IsoTag-AAV is remarkably robust, capable of >98% capture across a wide range of serotypes (2, 5, 8, 9 and rh10), platform across a 3-log range in titer (5E10-1E13 vg/ml) and can be completed in under 4 hours regardless of starting culture volume. Our process yields highly pure AAV whose *in vitro* infectivity is comparable or exceeds AAV purified by affinity chromatography. IsoTag-AAV actually improves upon traditional affinity yields and product quality with minimal optimization. A head-to-head comparison of IsoTag-AAV purification and traditional affinity purification at the 1L scale. This comparison showed yield improvements up to 20% unoptimized and comparable purity in half the time. Process cost modeling showed that IsoTag-AAV can reduce cost of goods for a commercial AAV program by as much as 50%. In conclusion, IsoTag™ AAV offers improved yields and comparable purity to traditional chromatography methods in a TFF process that scales linearly to streamline process development, scale up and manufacturing, accelerating time to the clinic

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics I

25 Focused Ultrasound for Improved Delivery of AAV Vectors to the Brain for the Treatment of Huntington's Disease

Nick Todd[1], Yongzhi Zhang[1], Lilyan Garrett[2], Alvin Yao[3], Kai Shing[4], Kimberly Kegel-Gleason[4]
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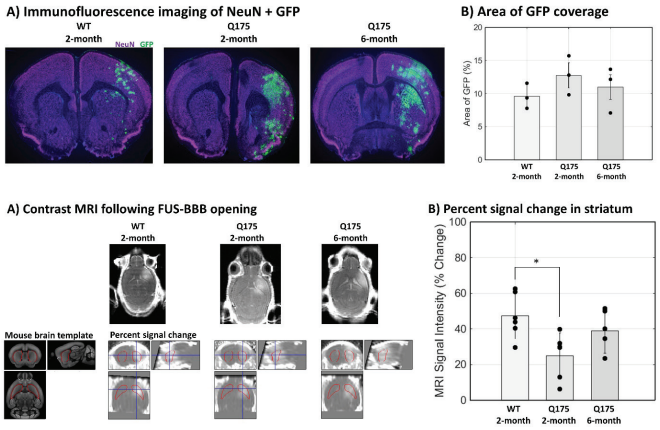
Background The goal of this study is to use the technology of focused ultrasound (FUS) to improve the delivery of gene therapies to the brain for the treatment of Huntington's disease. Pulsed FUS combined with systemically circulating microbubbles can achieve non-invasive, transient and spatially targeted opening of the blood-brain barrier (BBB) to facilitate delivery of normally non-penetrant agents into the brain. FUS is applied through the intact skull, can be focused to a region of only several millimeters, and can reach both cortical areas and sub-cortical structures. Gene therapies that knock down expression of the mutant Huntingtin protein are promising approaches to treat Huntington's disease. However, all gene therapies for brain disorders face the interrelated challenges of delivering the therapy across the BBB, and achieving an adequate therapeutic exposure at the targeted site while minimizing toxicity in healthy brain tissue and other organs. FUS-BBB opening may be an approach to improving the delivery of gene therapies over more invasive methods of intrathecal injection or direct neurosurgical injection currently used. **Methods** This study compares FUS-mediated delivery of an AAV9 capsid with green fluorescent protein (AAV9-GFP) in three mouse groups (N=6 per group): 2-month old wild type (WT) mice, 2-month old Q175 mice (mouse model of Huntington's disease), and 6-month old Q175 mice. FUS-BBB opening was targeted to the right striatum. Microbubbles were given by tail vein injection (Definity, 20 µL/kg) and FUS sonications were applied at 10 ms bursts and 1 Hz repetition frequency over 120 seconds. AAV9-GFP was given by tail vein injection immediately after FUS treatment (7.0e11 vg/mouse or ~2.8e10 vg/g) and contrast MRI images were acquired to quantify the extent of BBB disruption. Mice were sacrificed 3 weeks after treatment for immunohistochemistry. Brains were sectioned with Vibratome LEICA VT 1000 S and striatum sections were selected for fluorescent staining with GFP + [NeuN, GFAP, Iba1]. Sections were imaged using fluorescence microscopy and processed in MATLAB to quantify area of transfection. **Results** Figure 1 shows quantification of BBB opening based on contrast MRI images that are coregistered to a mouse brain template, converted to percent signal change and pixel values averaged over the full volume of striatum. One-way ANOVA indicates a significant main effect over the mouse groups ($F = 5.26$, $p = 0.019$, n=6 per group), with post-hoc comparison showing significant difference between WT and 2-month Q175 mice ($p = 0.015$) and a non-significant trend of difference between 2-month and 6-month Q175 mice ($p=0.14$). Figure 2 shows immunofluorescent imaging results from the first three mice in each group that have been processed to date. Significantly more GFP signal is seen in the FUS-targeted right

hemisphere compared to the non-targeted left hemisphere. Statistics to determine significant differences between mouse groups will be performed once all data sets are processed.



26 Liver-Specific Targeting CRISPR/Cas9 mRNA LNPs Achieve Long-Term FVIII Expression in Hemophilia A Mice

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Deficiency of X-linked factor VIII (FVIII) gene which participates in the intrinsic pathway of the coagulation cascade leads to a serious bleeding disorder, hemophilia A (HemA). Current treatment of HemA patients is routinely infused with FVIII protein several times every week as prophylactic treatment, which is costly and inconvenient. Gene therapy represents a very promising alternative treatment method. Combination of lipid nanoparticle (LNP) technology and CRISPR/Cas9 technique allow *in vivo* gene correction of the mutant FVIII. We aimed at optimizing *in vivo* delivery efficiency of mRNA encapsulated LNPs to repair the mutant FVIII gene and help HemA patients regain endogenous FVIII expression. Since FVIII protein is mainly and naturally made in liver sinusoidal endothelial cells (LSECs), we investigated various formulations of LNPs that can target LSECs. To examine the *in vivo* transfection efficiency of LSECs, we encapsulated luciferase (Luc) mRNA and GFP mRNA into MC3-based LNPs and delivered into mice, respectively. The efficacy of Luc mRNA LNPs and GFP mRNA LNPs was evaluated using *in vivo* imaging systems (IVIS) and immunofluorescent staining of the liver sections, respectively. Results suggested that this MC3-based LNP formulation can target both hepatocytes and LSECs *in vivo*. An immunodeficient hemophilia A (NSG HA) mouse model that contained frameshift-mediated premature stop codon in exon 1 of FVIII gene was used in this study. According to the *in vitro* gene editing data, we selected two efficient sgRNAs (mF8sgRNA and NSGHAsgRNA) for *in vivo* gene editing of mutant FVIII. We encapsulated Cas9 mRNA with each sgRNA into LNPs and intravenously deliver the LNPs to NSG HA mice, respectively. Endogenous FVIII activity was evaluated by aPTT assay and improvement of coagulation activity was examined by rotational thromboelastometry (ROTEM) assay. The Cas9 and FVIII-targeting sgRNAs LNP-treated NSG HA mice showed FVIII expression (1-7%) for more than 6 months. Moreover, we isolated the hepatocytes and LSECs from treated mice and the gene editing efficiencies of these two major liver cell types were verified by deep sequencing. The results showed that total percentages of frameshift mutation are 4.75% and 3.1% in hepatocytes and LSECs, respectively. Among all the frameshift mutations, percentages of one base deletion which can correct the frameshift-mediated nonsense mutation in NSG HA mice are 3.71% and 2.45% in hepatocytes and LSECs, respectively. All the data suggested that our LNP formulations can deliver mRNA into both hepatocytes and LSECs *in vivo*. Furthermore, combined with CRISPR/Cas9 technology, we have corrected the mutated FVIII gene in LSECs and regained the FVIII activity in NSG HA mice. This gene editing protocol shows the potential to persistently achieve therapeutic levels of FVIII gene expression for hemophilia A treatment.

27 A Novel Multitargeted Peptide-siRNA Complex for Simultaneous Inhibition of SARS-CoV-2 Host Cell Entry and Intracellular Viral Replication

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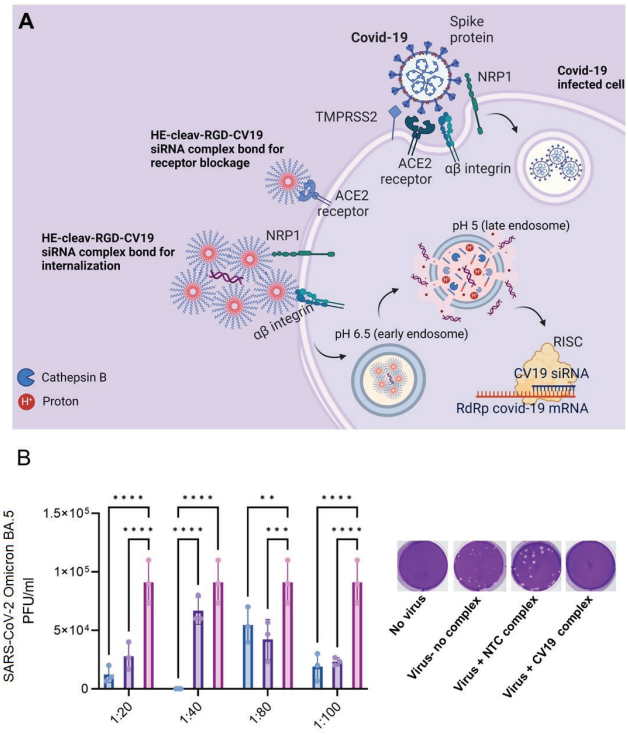
Although several successful vaccines have been developed against SARS-CoV-2, responsible for the COVID-19 pandemic, novel variants develop quickly and escape the vaccine protection while an effective and specific SARS-CoV-2 inhibitor is still missing. A promising approach is to silence a viral mRNA by administering a siRNA against the RNA-dependent RNA-polymerase (RdRp). Due to their limited blood stability and intracellular bioavailability, siRNA molecules must be combined with a vehicle. Cell-penetrating peptides are flexible vehicles that can be designed to allow spontaneous siRNA encapsulation and specific receptor binding. Peptides containing the RGD moiety interact with integrins and have been employed to target cancer cells. SARS-CoV-2 primarily enters the host cells by the interaction of viral spike protein with human angiotensin-converting enzyme 2 (ACE2). Moreover, human neuropilin-1 (NRP1) and some human integrins are also involved in the virus cell entry. We designed a novel RGD-containing self-assembling peptide (HE-cleav-RGD) to block SARS-CoV-2 by a combination of mechanisms (Figure 1A). The peptide interacted *in vitro* with recombinant viral spike receptor binding domain (spike RBD), human ACE2, human neuropilin-1, and spike-binding human integrins ITGAV and ITGA2. Moreover, the interaction of spike RGB with ACE2, ITGAV, and ITGA2 was displaced by the addition of HE-cleav-RGD. Furthermore, HE-cleav-RGD spontaneously encapsulated siRNA and protected its RNase degradation in serum. In Vero cells, GAPDH siRNA-HE-cleav-RGD complex allowed efficient GAPDH silencing in a dose-dependent manner that was inhibited by the addition of chlorpromazine (chlatrin inhibitor) and dynasore (dynamin inhibitor), suggesting that complex uptake occurs by receptor-mediated endocytosis. Further, immunocytochemical staining of Vero cells revealed colocalization of a fluorescent siRNA-HE-cleav-RGD complex with ITGAV, suggesting that the internalization was triggered by integrin binding. Finally, an

RdRp-targeting COVID-19 siRNA was complexed with HE-cleav-RGD and administered to Vero cells infected with ancestral or omicron BA.5 SARS-CoV-2. Plaque assay revealed that non-targeting siRNA complexed with HE-cleav-RGD (NTC siRNA-HE-cleav-RGD) allowed partial SARS-CoV-2 replication due to the inhibition of SARS-CoV-2 cell entry by the peptide. When HE-cleav-RGD is combined with an RdRp-siRNA (COVID-19 siRNA-HE-cleav-RGD), up to 100% inhibition of viral replication was observed at a siRNA-peptide molar ratio 1:40 (Figure 1B). Interestingly, SPECT scan of mice administered with I^{125}-labelled HE-cleav-RGD peptide revealed substantial lung accumulation. Altogether, our results suggest that HE-cleav-RGD peptide may be employed to develop promising anti-COVID-19 therapeutics.



28 Isolation of Natural and Biologically Active EV-AAVs for Brain Gene Delivery

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Adeno-associated viral (AAV) vectors are one of the preferred options for delivering genes to the central nervous system (CNS). Recent studies have shown that they can also be secreted along with extracellular vesicles (EVs) during production. This combination, known as EV-AAVs, improves AAV transduction in various organs, including the brain. This is due to the fact that EVs are naturally formed cell vesicles that can transport biomolecules and circulate without being targeted by the immune system. Despite the remarkable results obtained in previous reports, only AAV serotypes with already known ability to cross the blood-brain barrier have been used.In this work, we aimed at developing a size exclusion chromatography (SEC) protocol for the isolation of natural and biologically-active EV-AAVs from any AAV serotype for non-invasive gene delivery to the CNS. We also sought to compare EV-AAVs isolated by differential centrifugation (UC) and SEC. The EV-AAVs were produced following the standard protocol for AAV production and subsequently isolated by UC and SEC. Characterisation was performed by western blotting, nanoparticle tracking analysis (NTA), qPCR and transmission electron microscopy (TEM), while infectivity was evaluated *in vitro* and *in vivo.*The results indicated that SEC provides a higher recovery of natural EV-AAVs compared to UC (about four times higher), free from cell-contaminating proteins and with fewer solo AAVs. The SEC-purified EV-AAVs were also more infectious *in vitro* than solo AAVs. Upon intravenous injection in mice, SEC-purified EV-AAVs were able to cross the blood-brain barrier and the blood-cerebrospinal fluid barrier without AAV's contribution, and to successfully transduce mouse brains.In conclusion, SEC-purified EV-AAVs have the potential to be an effective gene delivery vector for the treatment of brain disorders.

29 Shuttle Peptides-Mediated Local RNP Delivery of Editing Complex in the Sensory Organs of the Inner Ear and Retina *In Vivo* in Adult Mice

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Genetic hearing loss and blindness are prevalent with limited treatment options. Despite the tremendous progress in the development of editing therapy for hearing loss and blindness, the ability to deliver the editing complex into the inner ear and retina remains the major challenge to realizing the potential. For viral vectors, issues like immunogenicity, genome integration and long-term effect remain a safety concern. In addition, with the more complex editing strategies including base editing and prime editing, multiple components need to be packaged into multiple AAVs, further limiting their efficiency. We have developed an amphiphilic S10 shuttle peptide and demonstrated delivery efficiency of CRISPR-associated nuclease ribonucleoprotein (RNP) to the respiratory tract of mice. In this study, we screened additional peptides of different charges and lengths to evaluate the application of shuttle peptides to deliver editing RNP complex into the sensory organs of the inner ear and retina in adult mice by local injection *in vivo*. The shuttle peptide-RNP formulation was delivered locally into the inner ear through the round window membrane (RWM) and retina by subretinal injection in adult Ai14 mice with tdTomato (tdT)

reporter. We identified shuttle peptides capable of RNP delivery into multiple inner ear sensory epithelial cell types and retinal cells with efficient editing. In the inner ear, the shuttle peptides mediated efficient delivery and editing in the supporting cell subtypes throughout the entire cochlear turn. Damage to outer hair cells was detected as a result of the delivery. In the retina, the shuttle peptides mediated efficient delivery and editing in the retinal pigment epithelial (RPE) cells and Muller cells, including those at a distance from the injection site. Muller cells at the injection site were also edited after injection. Using a shuttle peptide mixed with fluorescent cargo Cy5-NLS for the delivery into the inner ear, we detected Cy5 labeling in the majority of inner ear sensory epithelial cell types in adult mice, suggesting further modifications may target more inner ear cell types. The identification of shuttle peptides capable of efficient *in vivo* RNP delivery of editing agents in the mature mammalian inner ear and retina opens a new avenue to develop editing therapy for human hearing loss and blindness.

30 Hematopoietic Stem and Progenitor Cell Specific Antibodies and Intraosseous Administration Enhance Delivery of a CRISPR Nanoformulation

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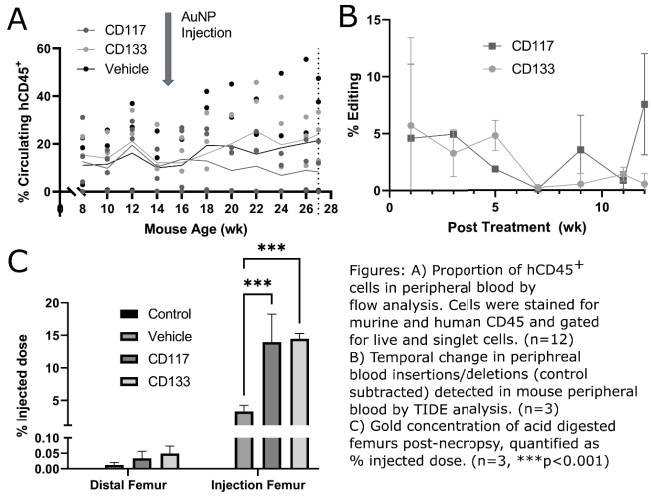
Hematopoietic stem and progenitor cells (HSC) have been a target for CRISPR gene editing (CGE) to treat multiple diseases. State of the art for HSC CGE occurs *ex vivo*, however, these methods remain challenging for global dissemination. *In vivo* delivery of CGE could facilitate global access but is inhibited by the rare and diffuse composition of HSC throughout the bone marrow niche. We previously described a layered gold nanoformulation to deliver CRISPR *in vitro* without electroporation (CR-AuNP)[1]. Here we sought to test this CR-AuNP *in vivo* in a humanized murine model of hematopoiesis[2]. One method to access HSC *in vivo* is to administer mobilizing agents, which reversibly increase HSC concentrations in peripheral blood. Initial experiments in mice (n=3) showed no toxicity associated with a single intravenous injection of CR-AuNP under mobilization, but only transient CGE (5.0 ± 2.5%) in peripheral blood leukocytes at 2 weeks after administration. To improve HSC-specific editing, we designed actively targeted CR-AuNPs, consisting of a conjugated targeting moiety. Optimal HSC targeting moieties were selected by winnowing a set of 22 known antibodies, aptamers and ligands for observed internalization by confocal microscopy within 1 hour of addition to primary human $CD34^+$ HSC. Three antibodies (αCD133:7, αCD117:104D2 and αCD90:5E10), two known aptamers (A15, B19), and one ligand (SCF) were identified for further testing. Whole bone marrow from six biological replicates was analyzed by flow cytometry to determine moiety specificity. αCD133 and αCD117 showed the highest specificity of 56.9 ± 7 and 66.4 ± 1% of $CD34^+/CD38^-$ cells, respectively, vs. 6.87 ± 0.25 and 1.17 ± 0.18% of total $CD34^-$ cells and were then loaded onto CR-AuNP via electrostatic binding. The resulting particle had a hydrodynamic diameter of 114.3 nm with a polydispersity index of 0.147 by dynamic light scattering. These particles were injected intraosseously into one femur of humanized mice (n=12), to increase local concentration within the bone marrow. Mice did not show any signs of toxicity by weight measurements or behavior by daily visual inspection. αCD117-CR-AuNP resulted in peripheral blood CGE of 4.6 ± 6.5% at week 2 as measured by Sanger sequencing and TIDE analysis, which persisted to 7.55 ± 4.5% at 12 weeks post injection (Figure B). Gold tracing by inductively-coupled plasma mass spectrometry at necropsy showed 14.2 ± 0.81% of Au dose within the injected femur ($p<0.001$ over bare AuNP control), with enhanced retention of targeted CR-AuNP in the distal femurs (Figure C). This data suggests CR-AuNP is well-tolerated *in vivo*, active targeting and intraosseous injection increases bone marrow retention, and this combination is associated with persistent CGE of peripheral blood leukocytes up to 12 weeks after administration in humanized mice.



Figures: A) Proportion of $hCD45^+$ cells in peripheral blood by flow analysis. Cells were stained for murine and human CD45 and gated for live and singlet cells. (n=12)
B) Temporal change in peripheral blood insertions/deletions (control subtracted) detected in mouse peripheral blood by TIDE analysis. (n=3)
C) Gold concentration of acid digested femurs post-necropsy, quantified as % injected dose. (n=3, ***p<0.001)

1. Shahbazi R, et al. Targeted homology-directed repair in blood stem and progenitor cells with CRISPR nanoformulations. *Nat Mater*. 2019;18:1124-1132. 2. Haworth KG, et. al. In Vivo Murine-Matured Human CD3+ Cells as a Preclinical Model for T Cell-Based Immunotherapies. *Mol Ther - Methods Clin Dev*. 2017;6:17-30.

31 NHEJ Gene Editing of Hemophilia A Mice Show Therapeutic Levels of FVIII Following Ultrasound Mediated Gene Delivery of CRISPR/Cas9 Plasmid

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Hemophilia A (HA) is a blood coagulation disorder, where an individual does not produce functional factor VIII (FVIII) protein. Treatment of this disease relies on repeated injections of FVIII, which is both costly and inconvenient for the patient. Correction of the mutation responsible for HA through CRISPR/Cas9 gene editing is a potentially permanent rescue of FVIII protein production that could avoid multitudinous treatments. We have previously

demonstrated that ultrasound mediated gene delivery (UMGD) can be a safe and effective non-viral method of targeting liver sinusoidal endothelial cells (LSECs), the native production site of FVIII, *in vivo*. In this study, we investigated the efficacy of combined ultrasound and microbubbles to successfully deliver CRISPR/Cas9 plasmids to HA mice for restoration of FVIII through gene editing. For the gene editing animal model, we used immunodeficient HA (NSG HA) mice with a 5 base pair deletion in FVIII exon I. RN18 microbubbles (MBs) and Cas9 plasmid containing an exon 1 targeting sgRNA (mF8sgRNA) were injected into the mouse portal vein for 30s, while simultaneously a pulsed, therapeutic ultrasound (US) transducer at 1.1 MHz frequency and 14 Hz PRF treated the liver surface for 60 s at 50 W, 150 µs PD. This US condition has previously been demonstrated to predominantly transfect LSECs. The FVIII activity was determined via the aPTT assay at various timepoints over 90 days, and whole blood clotting was evaluated via ROTEM. Livers were sectioned, stained, and imaged to examine production of FVIII mRNA using RNAscope© Multiplex Fluorescent staining assay. Successful gene editing was verified by DNA sequencing. Factor VIII activity levels for treated NSG HA mice, n=7, averaged 5% over 90 days (Figure 1). Increased production of FVIII mRNA was detected in the liver sections of treated mice compared to untreated naïve mice, in addition to colocalization of FVIII and Lyve-1 (LSEC marker) signals indicating targeting of LSECs. The ROTEM assay showed an improvement in clotting time and clot formation time (Figure 2), signifying therapeutic correction of the severe HA phenotype. DNA sequencing confirmed successful induction of indels with an editing efficiency of approximately 4.3%. UMGD successfully delivered a CRISPR/Cas9 plasmid to livers of NSG HA mice to induce a mutation in the target site, resulting in therapeutic levels of FVIII expression. This indicates UMGD is a potentially effective non-viral technique for delivering gene editing tools *in vivo* for the treatment of HA.

Figure 1. NSG HA mice treated with UMGD had hFVIII activity levels of approximately 5% over 90 days

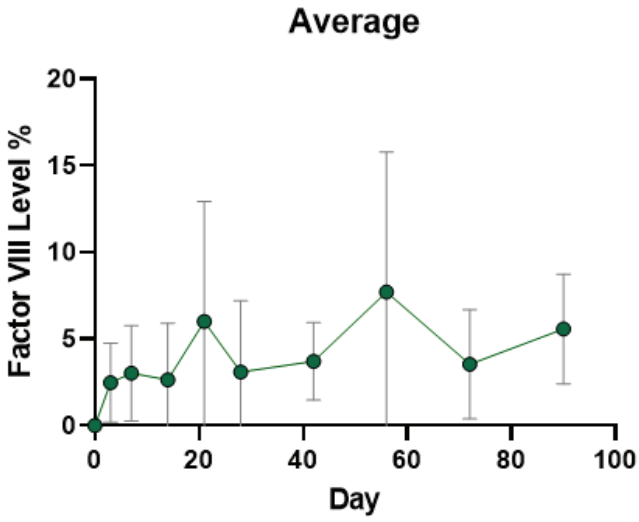
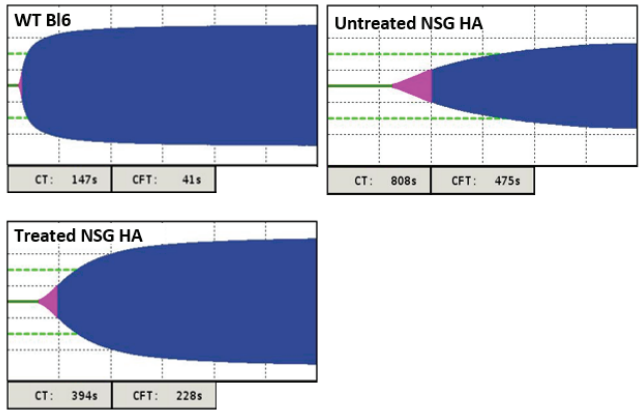


Figure 2. ROTEM blood clotting assay done in wild type BL6 mouse, untreated NSG HA mouse and UMGD treated NSG HA mouse displaying clotting time (CT) and clot formation time (CFT)



Epi-/Genetic Editing and New Methods to Target the Nervous System

32 Zinc Finger Transcriptional Regulator Mediated Repression of *SCN9A* as a Therapeutic Approach for Painful Peripheral Neuropathies

Mohammad Samie, Toufan Parman, Mihika Jalan, Jisoo Lee, Patrick Dunn, Jason Eshleman, Josh Holter, Sarah Hinkley, Alicia Goodwin, Dianna Vidales, Dan Chung, Luke Workley, Kenneth Kennard, David Ojala, Matt Tiffany, Sandeep Yadav, Bryan Zeitler, Jason Fontenot, Amy Pooler

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Painful peripheral neuropathies are caused by severe and permanent damage to peripheral sensory or motor neurons and are estimated to affect several million patients in the US alone. Given the high unmet need and lack of effective treatments, there is an urgent need to develop novel therapeutics for the treatment of chronic painful peripheral neuropathies. The voltage-gated sodium channel Nav1.7, encoded by the *SCN9A* gene, is involved in a spectrum of inherited human pain disorders. Loss or gain of function mutations in *SCN9A* result in insensitivity or hypersensitivity to pain, respectively, highlighting the central role of Nav1.7 in pain transmission to the brain. Development of selective and efficacious Nav1.7 inhibitors has been challenging due to similarities in protein sequence and structure among the Nav channels. We hypothesized that lowering Nav1.7 via selective repression of the *SCN9A* gene using engineered zinc finger repressors (ZF-Rs) would reduce the expression of Nav1.7, and therefore reduce neuropathic pain. To evaluate the feasibility of this approach, ZF-Rs specifically targeting the mouse *Scn9a* gene were designed and assessed *in vivo* in a neuropathic pain spared never injury (SNI) mouse model. AAV-mediated ZF-R delivery resulted in up to 70% bulk repression of *Scn9a* in mouse dorsal root ganglion (DRG) tissue. *Scn9a* repression was associated with significant reduction in pain one month post-treatment as measured by increased mechanical and cold allodynia threshold in the SNI model of neuropathic pain. The repression of *Scn9a* was

not associated with neuroinflammation or neuronal loss in DRGs. To identify human ZF-R candidates, a library containing more than 300 ZF-Rs targeting the human *SCN9A* gene was designed and screened in human neuronal cells lines. Lead ZF-Rs were packaged into AAV and tested in human iPSC-derived GABAergic and iPSC-derived sensory neurons *in vitro*. We identified ZF-Rs that repressed *SCN9A* by more than 90% over a wide dose range with no detectable off-target activity as measured by global transcriptome analysis, including selective repression of Nav1.7. Candidate human ZF-Rs are being evaluated for pharmacology and safety in nonhuman primates. Taken together, our results support the development of AAV-delivered ZF-Rs for the treatment of painful chronic neuropathic indications.

33 Delivery of BDNF through a Pluripotent Stem Cell-Based Platform Ameliorates Behavioral Deficits in a Mouse Model of Huntington's Disease

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Huntington's disease (HD) is a life-threatening neurodegenerative disease that occurs due to an autosomal dominant mutation of CAG trinucleotide repeat expansion beyond 35 repeats in exon 1 of the Huntingtin (*HTT*) gene. The expanded CAG repeats encode for a mutant HTT with an abnormally long polyglutamine tract. This mutant HTT forms toxic aggregates in the brain which inhibit neuronal function and survival to cause neurodegeneration. Striatal medium spiny neurons (MSN) are the most affected and neuronal subpopulations of the cerebral cortex are affected to a lesser extent. Current treatments for HD are only palliative and do not stop or reverse the neurodegeneration. Studies in animal models have shown that viral vector or cell-based delivery of secretory neuroprotective (NP) factors ameliorates HD-associated neurodegeneration. However, host immunity against viral vectors and transplanted allogeneic or xenogeneic cells poses a significant barrier for clinical translation of this approach. To address this, a pluripotent stem cell (PSC)-based delivery platform could be utilized due to the feasibility of deriving large scale therapeutically relevant non-immunogenic autologous cell types. The goal of this study is to test the feasibility of using a PSC-based platform to deliver NP factors in the brain to treat HD. Here, we have tested the therapeutic potential of delivering BDNF and NT-3 NP factors using a PSC-based platform in the R6/2 HD mouse model. For this, we have used a previously generated H9 embryonic stem cell line expressing a rapamycin-inducible safety switch transgene. For the expression of NP factors, we performed gene targeting in PSC at the HBB locus, a safe harbor for neural cell types. For this HBB gene targeting, we used gene editing with Cas9 ribonucleoprotein (RNP) and AAV6 based homology directed repair donor template delivery to express NP factors from a constitutive promoter. Post-gene editing, we isolated single cell PSC clones with bi-allelic gene targeting of BDNF, NT-3 and confirmed the protein secretion *in vitro*. Using previously described differentiation protocols, we derived neural and striatal progenitors from the gene edited PSC for the delivery of NP factors in the brain. To assess the therapeutic potential, we performed intra-striatal transplantation of the PSC derived neural and striatal progenitors in the R6/2 HD mouse model following antibody-based immunosuppression. Post-transplantation, motor and cognitive functions were assessed through nest building, rotarod, marble bury, open field and social memory behavior tests. Neural progenitors with and without NT-3 expression ameliorated deficits in some of the behavior tests when compared to the vehicle injected R6/2 mice. Neural progenitors expressing BDNF significantly ameliorated the deficits in all the behavior tests indicating improved motor and cognitive functions. Striatal progenitors expressing BDNF moderately ameliorated various behavioral deficits. Upon histology analysis, we found that the transplanted neural and striatal progenitors can differentiate into post-mitotic neurons expressing NeuN marker *in vivo*. Importantly, we have validated the feasibility of activating the safety switch to eliminate the transplanted cells in the brain following intraperitoneal injection of rapamycin. Thus, delivery of BDNF through PSC derived neural progenitors could an effective therapeutic intervention for HD and the applicability of safety switch enhances the safety of this approach.

34 RNA Editing Therapy in a Humanized Mouse Model of MECP2 Duplication Syndrome and Non-Human Primates

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Background: MECP2 duplication syndrome (MDS) is a rare, fatal childhood neurodevelopmental disorder with progressive neurological regression such as loss of motor skills and speech, predominantly affecting boys and accounting for about 1% of X-linked cases of intellectual disability. MDS is caused by the duplication of the methyl-CpG binding protein 2 (*MECP2*) gene which locates at the chromosome Xq28. To date, there is no effective treatment for this devastating disease. **Methods**: We tested the treatment efficacy of AAV delivering the system of high-fidelity Cas13Y (hfCas13Y, a high-efficiency and high-fidelity RNA editor) and gRNAs targeting MECP2 (gMECP2) (AAV-hfCas13Y-gMECP2), in postnatal day 0-1 male humanized MDS mice (from the Jackson Laboratory) via intracerebroventricular (ICV) injection (Fig. 1A). Additionally, we validated the knockdown efficiency of *MECP2* in the brain of wild-type non-human primates (NHPs) via ICV administration of AAV-hfCas13Y-gMECP2 (Fig. 1H). The behavioral tests were performed in humanized MDS transgenic mice injected with AAV-hfCas13Y-gMECP2 and their controls, including accelerating rotarod, open field, elevated plus maze, and 3-chamber social tests. **Results**: The hfCas13Y and *MECP2* gRNAs were stably expressed in brain regions of mice and monkeys where the pathological changes of MDS mainly occur. Furthermore, the expression of MECP2 was knocked down to 40-60% and almost reversed to the normal level (wild-type mice level) in the brain of humanized MDS mice (Fig. 1B,C). Importantly, the accelerating rotarod shows that the prolonged latency to fall from the rotating rod decreased in AAV-hfCas13Y-gMECP2

treated MDS mice (Fig. 1D). The open field test shows that the MDS mice displayed reduced exploratory behavior, which could be rescued by the treatment of AAV-hfCas13Y-gMECP2 (Fig. 1E). Moreover, the elevated plus maze test shows that the anxiety-like behavior of MDS mice was increased compared with wildtype (WT) mice, and it could be reversed to normal level (WT mice) by the administration of AAV-hfCas13Y-gMECP2 (Fig. 1F). In the 3-chamber social test, the MDS mice showed less communication behaviors, which could be rescued to normal level by the treatment of AAV-hfCas13Y-gMECP2 (Fig. 1G). Similar to the humanized MDS mice and in the brain of monkeys, the MECP2 knockdown efficiency was more than 20 % in 40 % brain regions, and among which the highest one reaches 70% compared with the control (Fig. 1I). In addition, there was no *in vivo* toxicity (including nerve, liver, and kidney injury) observed in the humanized MDS mice and monkeys injected with the high dose of AAV-hfCas13Y-gMECP2 administration. **Conclusions:** Since the AAV we used is able to cross the blood-brain barrier and to infect neurons efficiently, our findings provide a strong foundation and promising approach for the clinical application of AAV delivering hfCas13Y-gMECP2 in MDS, which may potentially improve the motor and behavioral impairment in patients with MDS.

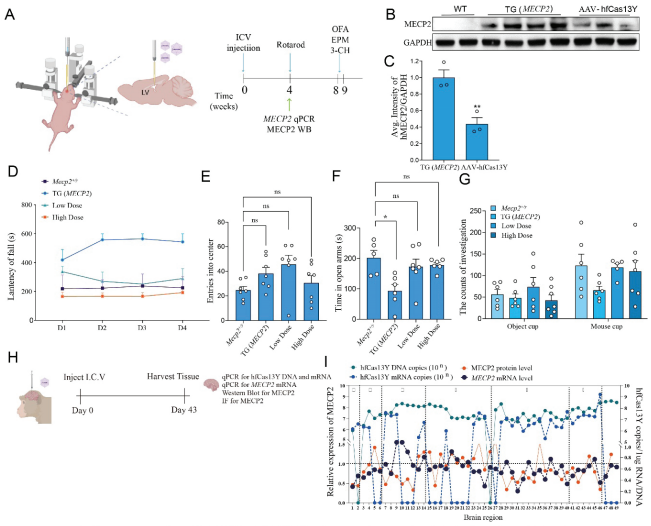

Figure 1. The treatment effect of AAV-hfCas13Y-gMECP2 in the male humanized MDS mice and the WT monkey

35 The Interplay between Central Nervous System and Peripheral Organs: The Lesson from the Treatment of Canavan Disease with rAAV Gene Therapy

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Mutations in the Aspartoacylase (ASPA) gene lead to a lethal neurodegenerative condition called Canavan disease (CD), which is characterized by progressive neurodegeneration with dysmyelination and accumulation of N-acetylaspartate (NAA), the only substrate of ASPA in oligodendrocytes. In the CD knock-out (KO) mouse model, with the most severe disease pathology and early death at around postnatal day (P) 27, we previously showed complete disease prevention with systemic delivery of ASPA by recombination adeno-associated virus 9 (rAAV9ASPA). However, central nervous system (CNS)-restricted gene therapy by intracerebroventricular (ICV) injection could not completely prevent the phenotypes compared to those with intravenous (IV) treatment despite similar brain biomarkers in both groups and equivalent ASPA enzyme activity. Therefore, we hypothesized that peripheral organs, such as liver, could mediate this effect. We used neuro-metabolomics and transcriptomics to explore this connection to analyze the brain and peripheral organs (serum) in WT and CD mice. Two key findings were the elevation of ketone body β-hydroxybutyrate (bOHB) and several inflammation markers in the CNS. We hypothesized that treatment of peripheral organs might modulate CNS metabolism and immunopathology. To test this hypothesis, we treated neonatal CD KO mice with rAAV9ASPA with cell-type specific promoters: neurons, astrocytes, oligodendrocytes, heart, liver, and skeletal muscle. To our surprise, mean overall survival was substantially improved in the liver-restricted group without normalized brain NAA levels. Interestingly, despite liver-restricted restoration of ASPA protein, the liver function was only partially rescued, i.e., bOHB pathway, compared to the wildtype mice, suggesting that extrahepatic factors contribute to the biochemical liver profile. Due to bOHB's known function in epigenetic regulation, we next tested if bOHB modulates histone modifications in the CNS and found that liver-restricted ASPA expression normalized several histone modifications by bOHB and acetyl-CoA in the brain and liver - a finding we are currently investigating. bOHB and epigenetic mechanisms are important modulators of the immune response, and our transcriptomics data indicated differential expression of several genes in the CNS immune response. Furthermore, interrogation of several of these genes showed that liver-restricted expression changed some proteins profiles in the brain, suggesting that liver treatment has CNS immune modulatory effects that might modulate the disease progression favorably In sum, this study provides novel insight into the disease-modifying role of peripheral organs on CNS pathology in Canavan disease rAAV gene therapy through biochemical and immunomodulating function with possible implications for other CNS-targeting gene therapies.

36 Gene Editing Strategies to Treat Spinocerebellar Ataxia Type 1

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Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that causes progressive loss of motor coordination, respiratory issues and eventual death. SCA1 is caused by expansion of the polyglutamine repeat region in the ATXN1 gene. Although mATXN1 is expressed ubiquitously, it affects primarily Purkinje cells (PCs). There are currently no treatment options for SCA1. We hypothesize that CRISPR-Cas editing of ATXN1 will reduce mutant ATXN1 and be therapeutically beneficial For Cas9, we designed two different strategies to reduce ATXN1; the first uses a single guide RNA (gRNA) to target near the exon-exon junction to induce nonsense mediated decay and the second approach employs a dual guide system to delete the CAG repeat region. In

cultured cells, the single guide approach reduced ATXN1 mRNA levels by 40-45% ($p \leq 0.02$) and protein by approximately 20% ($p \leq 0.01$) and the dual guide approach reduced levels of mRNA and protein levels by 70-75% ($p < 0.001$) and 45-65% ($p \leq 0.03$), respectively. For the dual guide strategy, DNA editing assessed by Sanger sequencing confirmed full length deletions at the expected size in vitro. For testing in vivo, SCA1 mice, expressing mutant human ATXN1 were injected with recombinant AAVs (rAAVs) expressing the optimized gRNAs from each strategy and spCas9. The viruses were co-delivered into neonatal mice with an intracerebroventricular injection for transduction of PCs. Both the exon-exon strategy and the dual guide strategy reduced mRNA ATXN1 levels by 55% ($p < 0.001$) and 40% ($p < 0.001$), respectively, compared to saline injected controls. Oxford Nanopore sequencing after in vivo delivery confirmed full length deletions. Further analysis is underway to more fully define the editing events and to assess the efficacy of the single and dual guide editing approaches on mice behavior and neuropathological readouts. In another strategy, we developed approaches for CRISPR-based RNA knock down using RfxCas13d. RfxCas13d is an RNA specific nuclease that binds to target mRNA and cleaves the sequence complementary to the gRNA. RfxCas13d guides designed to target ATXN1 transcripts reduced ATXN1 mRNA by 50% ($p < 0.001$) in cells and in short term pilot studies in B05 mice, reduced ATXN1 mRNA by 10% reduction ($p = 0.001$). Cumulatively, our work contrasts and compares several editing strategies for reduction of ATXN1 for SCA1 therapy.

37 Single-Cell RNA-Sequencing to Evaluate rAVV Transduction in the CNS and Its Therapeutic Implications for Canavan Disease Gene Therapy

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Gene therapy using recombinant adeno-associated virus (rAAV) vectors has rapidly emerged as one of the leading approaches for treating various inherited disorders of the central nervous system (CNS). Although the successful use of rAAV-based gene therapy in clinical trials are promising, two central questions remain poorly understood. 1) How effective are different cell types transduced via intravenous delivery in the CNS? and 2) Do successfully transduced cells exert therapeutic benefits on untreated cells in the CNS? We used Canavan disease (CD) as a model system to narrow this knowledge gap. CD is a lethal leukodystrophy caused by mutations in the ASPA gene, which encodes the enzyme aspartoacylase (ASPA). ASPA is mainly expressed in oligodendrocytes in the brain, metabolizing N-acetylaspartate (NAA) into L-aspartate and acetate. First, using the CD knock-out (CD KO) mouse model, mice treated intravenously at neonatal age with rAAV9-based gene therapy that we previously demonstrated can completely prevent the disease. Mice were sacrificed several weeks later to determine rAAV transduction and therapeutic efficacy in cellular resolution. We used a modified protocol to successfully isolate cells from the brains of treated (Tx), untreated (UT), and wild-type (WT) control mice and performed single-cell RNA-sequencing (scRNA-seq). In detail, we obtained 62,538 cells (WT: 18,155; CD-UT:20,297; CD-Tx:24,096) in total, with a mean 1906 transcripts detected per cell, using the 10X Genomics Chromium platform. With Seurat analysis, all of the primary cell types of the brain were captured on the Uniform Manifold Approximation and Projection (UMAP) plots, including excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, microglia, endothelial cells, fibroblasts. Further analysis revealed that all major cell types are successfully transduced and transcribed ASPA, including oligodendrocytes in the CD-Tx group. Moreover, we compared the differences between oligodendrocytes with or without transgene expression and the differences for each cell type among the WT, UT, and Tx groups to understand the functional consequences of ASPA transduction. This analysis allowed us to 1) reveal the transcriptomic differences for each cell type in the brain between WT, UT, and Tx groups, providing insight into the molecular mechanisms underlying CD; 2) "visualize" the therapeutic response to rAAV9-based gene therapy, and 3) assess the safety of this therapeutic approach by characterizing microglial response to rAAV9 and 4) demonstrating the transduction efficacy of rAAV9. Together, this study provides a comprehensive preclinical assessment of rAAV9 in the CNS after neonatal delivery and highlights the therapeutic relevance for Canavan disease, which could provide further insight to overcome the challenges of CNS-directed rAAV gene therapy.

38 Ionizable Lipid Nanoparticles for Therapeutic Base Editing of Congenital Brain Disease

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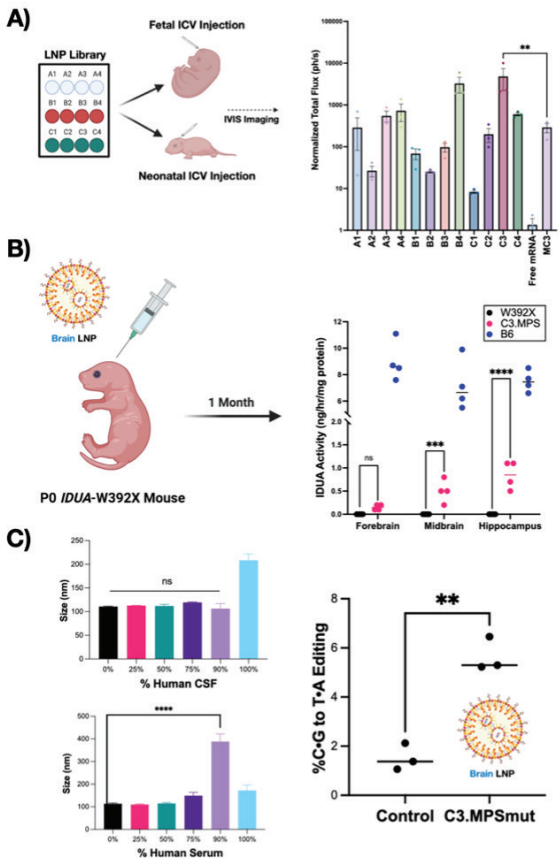
Introduction: Congenital neurologic diseases affect 1% of children at birth and account for 40% of pediatric hospitalizations. Editing the genome perinatally and correcting pathogenic mutations prior to disease onset represents a novel therapeutic strategy that could reduce neurodevelopmental symptoms associated with these disorders. A key challenge for translation of mRNA-based gene therapies is safe and effective intracellular delivery. While ionizable lipid nanoparticles (LNPs) are efficacious, biocompatible nucleic acid delivery vehicles, LNPs have not yet been engineered specifically for the delivery of nucleic acids to the perinatal brain environment. Here, we screened a diverse library of LNPs *in vivo* to target the fetal and neonatal mouse brain, optimized LNPs in cells of neural origin *in vitro*, engineered an LNP-base editing platform to correct the biochemical phenotype of mucopolysaccharidosis type-1 (MPS-I) in the neonatal mouse brain, and exhibited proof-of-principle delivery and safety in a fetal NHP and patient-derived brain tissues. **Materials and Methods:** LNPs encapsulating *luciferase* mRNA were formulated as described previously, characterized, and screened in BALB/c fetuses (E18) and neonates (P0) via intracerebroventricular (ICV) injection. The top performing LNP was optimized in a neuroblastoma cell line and primary murine MPS-I cells for excipient ratio, N:P ratio, and adenine base editor (ABE) to guide RNA (sgRNA) ratio. Optimized LNPs were combined with a base editing platform for murine MPS-I and injected ICV in MPS-I neonates.

After 1 month, mice were analyzed for on- and off-target editing, biochemical pathway rescue, and safety via cytokine panel and anti-PEG IgM response. Finally, optimized LNPs were injected ICV into a fetal NHP, assessed for stability in human cerebrospinal fluid (CSF), tested in patient-derived neurons, and used to base edit the human gene involved in MPS-I *ex vivo* in human precision cut brain slices. **Results and Discussion:** Our library screen revealed a novel LNP that mediates a 17-fold increase in mRNA expression in the fetal brain in comparison to an industry standard with similar results observed in the neonatal brain (Fig. 1A). LNPs optimized *in vitro* facilitated low-level brain editing *in vivo*, mediated biochemical rescue of disease - 7 to 13% of normal enzymatic activity - in the neonatal MPS-I mouse brain (Fig. 1B), and did not induce an innate or humoral immune response. Brain-optimized LNPs successfully transfected periventricular tissue of a fetal NHP, were stable in human CSF (Fig. 1C), transfected 90% of patient-derived neurons without toxicity, and facilitated base edits in human brain tissue. **Conclusions:** Our novel LNPs are highly effective at mRNA delivery to both the fetal and neonatal mouse brain. After optimization of formulation parameters *in vitro*, our LNP LNP-base editing platform demonstrated therapeutic efficacy and safety in a mouse model of MPS-I. These LNPs have high potential for clinical translation, since they transfected the fetal NHP brain in vivo, showed stability in human CSF, and are non-toxic and highly effective in transfecting patient-derived tissues. In the future, the LNPs developed in this study can be broadly applied for perinatal mRNA therapeutics targeting the CNS.



AAV Library Technology

39 Parallel Engineering and Recombination of Adeno-Associated Virus Variable Regions Enables Multisite Library Production

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Generation of diversified adeno-associated virus (AAV) capsid libraries and subsequent selections of variants has yielded exciting vectors with distinct tropism. However, one limitation of current engineered variants of AAV is that much of the surface-exposed variable regions from the parent are included in the final product, which could cause variants to maintain the receptor binding interactions of their ancestral sequence and contribute to neutralization by the immune system. While mutating surface-exposed loops iteratively allows for gradual alteration of the tissue tropism away from the parent sequence[1], this creates some dependency on previous mutations and restricts the accessible protein landscape for the capsid. With the aim of obtaining AAV capsids with minimal parental sequence present in the prominent surface-exposed loops, we engineered all three surface-exposed regions of the AAV9 capsid by 7-mer substitution. By screening capsid libraries for production, we mutated each of three surface-exposed variable regions of the three-fold axis prior to selection, enabling a wider search of the protein landscape. We then combined these pre-selected libraries for each site to yield capsids with all prominent, surface-exposed residues mutated. Using this approach, we engineered two capsid libraries, containing 14 and 21 amino acid substitutions, respectively, and found that our parallel strategy boosted production and yielded diversified libraries.

1. Goertsen, D., Flytzanis, N. C., Goeden, N., Chuapoco, M. R., Cummins, A., Chen, Y., ... & Gradinaru, V. (2022). AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset. Nature neuroscience, 25(1), 106-115.

40 APPRAISE: Fast, Accurate Ranking of Engineered Proteins by Receptor Binding Propensity Using Structure Modeling

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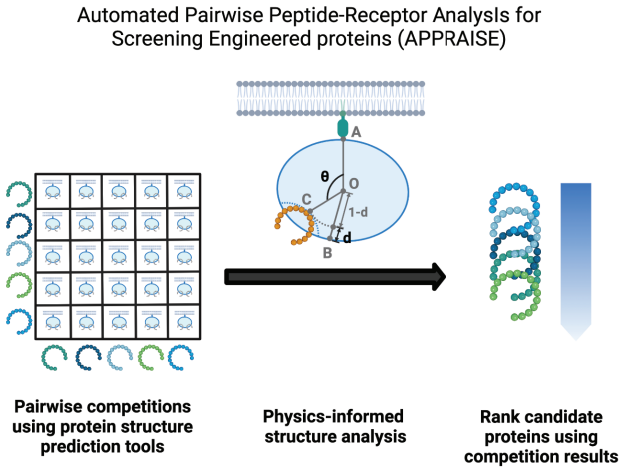
Deep learning-based methods for protein structure prediction have achieved unprecedented accuracy. However, the power of these tools to guide the engineering of protein-based gene & cell therapeutics remains limited due to a gap between the ability to predict the structures of candidate proteins and the ability to assess which of those proteins are most likely to bind to a target receptor. Here we bridge this gap by introducing Automated Pairwise Peptide-Receptor AnalysIs for Screening Engineered proteins (APPRAISE), a method for predicting the receptor binding propensity of engineered proteins. After generating models of engineered proteins competing for binding to a target using an established structure-prediction tool such as AlphaFold2-multimer or ESMFold, APPRAISE performs a

rapid (under 1 CPU second per model) scoring analysis that takes into account biophysical and geometrical constraints. As a proof-of-concept, we demonstrate that APPRAISE can accurately classify receptor-dependent vs. receptor-independent engineered adeno-associated viral (AAV) vectors, as well as diverse classes of engineered proteins such as miniproteins targeting the SARS-CoV-2 spike protein, nanobodies targeting a G-protein-coupled receptor, and peptides that specifically bind to transferrin receptor and PD-L1. When used to screen a library of 100 AAV variants, high-throughput APPRAISE (HT-APPRAISE) correctly predicted an AAV variant with a distinct sequence from previously known receptor binders to be a top receptor binder. The results of both in vitro and in vivo experiments showed that the top AAV variant has strong receptor-dependent transduction. We made APPRAISE accessible through a web-based notebook interface using Google Colaboratory (http://tiny.cc/APPRAISE). With its high accuracy, interpretability, and generalizability, APPRAISE has the potential to expand the utility of current structural prediction methods and accelerate protein engineering for gene & cell therapies.



41 *In Vivo* AAV Capsid Selection at Spatial and Single-Cell Resolution

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Differential regional and cellular vulnerability is a hallmark of neurodegenerative disease and a challenge for brain-targeting gene therapies. The development of novel adeno-associated viruses (AAVs) with tropism for disease-affected regions and cell types is crucial to therapeutic efficacy. AAV selection methodologies have given rise to a new class of engineered AAV vectors capable of improved gene delivery. Current AAV selection methods yield tissue-level enrichment readouts by way of universal promoters, or cell-type enrichment information via cell-type-specific promoters, over multiple rounds of enrichment. While this approach has proven fruitful, current methods are not capable of resolving transduction status in heterogenous cell populations across distinct tissue regions. Existing approaches for the detection of cell-type-specific tropism of engineered AAVs via single-cell RNA sequencing are low-throughput and/or cost-prohibitive for profiling many regions and biological replicates, in part, due to reliance on droplet-based single-cell or nuclei partitioning. To overcome these limitations and implement a scalable method for highly multiplexed profiling of regional and cell-type-specific AAV tropism, we utilized a combinatorial indexing method for single-cell transcriptome sequencing (Rosenberg et al. 2018). Top hits from a previous peptide-modified AAV screen, identified for widespread transduction of deep brain structures, were selected for a proof-of-principle experiment. A mixed pool of 62 top-performing capsids was delivered via direct injection to the striatum or thalamus of adult mice (N=2 mice per injection site). Tissue was collected from the site of injection, as well as other interconnected brain regions (cortex and hippocampus). In total, 12 tissue samples were processed simultaneously, from four mice and four brain regions, for multiplexed single-nuclei RNA sequencing. Custom computational pipelines were developed for the integration and analysis of AAV transduction profiles in the context of single-cell transcriptomes. Here we demonstrate successful capture of the regional and cellular origin of AAV transduction events. We then associate each event with the delivering capsid variant, allowing us to resolve which capsid variants transduced which cell types and in which tissue regions. In addition, we demonstrate the use of this technology to track AAV biodistribution across cellular networks and anatomical regions, a use case that can be applied to numerous biological contexts. This approach has many advantages for scalability. As of now, a single experiment can be expanded to profile ~150,000 cells or nuclei, from hundreds of biological samples, and can be used to assess 10s to 100s of AAV capsid variants simultaneously. Furthermore, our method requires no specialized equipment and can be applied across tissues and species.

42 Antibody Conjugated AAV Vectors for Efficient and Specific Skeletal Muscle Directed Gene Delivery across Species

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Regeneron Pharmaceuticals, Tarrytown, NY

Adeno-associated virus (AAV) is a leading viral vector used in in vivo gene replacement therapyand gene editing in preclinical and clinical studies. Although AAV has many advantages as a genetherapy vector, selective transduction of specific tissues after systemic delivery remains achallenge. Many AAV capsids preferentially transduce the liver after systemic injection, whichlimits the efficiency of transduction in other tissues and cell types. This is particularly challengingfor skeletal muscle as it comprises up to 40% of total body mass. To achieve therapeutic efficacyexceptionally high virus doses (>1E+14 vg/kg) are required. Several safety concerns associatedwith high dose systemic AAV therapies have been observed in some recent clinical trials,highlighting the need for more efficient AAV vectors that can achieve efficacy at lower doses.Although capsid library-based

selection approaches are a powerful method to identify novelcapsids with desired properties, the translatability of such capsids between species remains aconcern. We aimed to develop a species agnostic AAV retargeting approach that utilizes rational capsid engineering and monoclonal antibodies to redirect viral particles to skeletal muscles. We utilize a protein tagging system to covalently conjugate antibodies to surface-exposed variableloops on the viral capsid. The capsid can be further engineered through the introduction of pointmutations in the amino acid residues that serve as primary determinants of vector tropism. Formuscle targeting, we identified a protein, CACNG1, that is specifically expressed in skeletalmuscle. We also created humanized mice that are genetically modified to express the humanCACNG1 protein. We then generated panels of monoclonal antibodies raised against the humanCACNG1 protein to test in our retargeting platform. Here we demonstrate that AAV particles canbe retargeted to skeletal muscle in an antibody-dependent manner both in vivo and in vitro. Thepanel of CACNG1 Ab-retargeted AAV9 in vitro showed substantially enhanced reporter transgeneexpression in human skeletal muscle cells as well as mouse myotubes. The retargeted capsidsdisplay enhanced skeletal muscle delivery and marked liver detargeting compared to AAV9, astandard wildtype capsid used for muscle delivery in human clinical trials. The retargeted capsidsalso demonstrate enhanced delivery in mouse models of disease such as Duchenne musculardystrophy (D2-mdx) compared to AAV9. To demonstrate the cross-species translatability of ourplatform, we show that our antibody-targeted muscle-specific capsids outperform AAV9 whencompared head-to-head in non-human primates using a barcoded library approach. The extentof liver detargeting as well as the relative efficiency of the panel of antibodies tested were similarbetween rodents and cynomolgous monkeys, demonstrating the translatability of our platform.

43 Generative Networks Create Novel Receptor Targeted AAVs with Only 1,200 Training Examples

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Introduction: Searching vast sequence spaces for protein variants with enhanced biological functions of interest can be challenging when only a small percentage of variants meet these criteria. We investigated the feasibility of applying SeqDesign[1], a Wavenet-based generative neural network, to nominate adeno-associated virus (AAV) capsid variants given only a small sample of known high-performance candidates. **Methods:** Libraries of AAV9 capsid variants modified with 7-amino acid insertions at the surface exposed loop VIII were subjected to functional assays to evaluate their (1) production fitness and (2) ability to bind to a specific human brain endothelial cell surface protein in vitro. The data were used to train different generative models, which were assessed by the diversity and performance distribution of their predicted variants. **Results:** *Training size optimization:* We trained Wavenet models on 20k, 10k, 5k or 1k production-fit sequences and evaluated their performance using our previously established production-fitness model.[2] The predicted fitness of generated capsids decreased at 1,000 training sequences (Fig 1A). *Generating receptor targeted AAVs:* We trained a Wavenet model to predict variants capable of binding to a specific human protein present on the human CNS vasculature. We used 1,200 high-performance candidates identified in an in vitro target protein binding assay[3] for training. Wavenet's outputs were diverse; minimum self-Hamming distances were 1: 62%, 2: 30%, 3: 7.4%, 4: 0.4%. The output was novel compared to the training examples; training-to-generated minimum Hamming distances were 1: 16%, 2: 38%, 3: 37%, 4: 8.4%, 5: 0.04% (Fig 1B). *Validating generated capsids:* We designed a validation library using the receptor targeted variants generated by Wavenet. Wavenet performed well in the critical right-tail prediction task; its best outputs performed as well as variants previously measured in vitro (Fig 1C). **Conclusion:** We demonstrated that Wavenet could generate a highly diverse set of variants that were production-fit and bound the target human receptor. Importantly, in contrast to other ML models, Wavenet generated a larger number of rarer, ultra-high performing receptor targeted variants, which are prime candidates for further development as gene delivery vectors.

[1] Shin, Jung-Eun, et al. "Protein design and variant prediction using autoregressive generative models." Nature communications 12.1 (2021): 1-11. [2] Eid, Fatma-Elzahraa, et al. "Systematic multi-trait AAV capsid engineering for efficient gene delivery." bioRxiv 2022.12.22.521680. [3] Huang, Qin, et al. "Targeting AAV vectors to the CNS via de novo engineered capsid-receptor interactions." bioRxiv 2022.10.31.514553v1

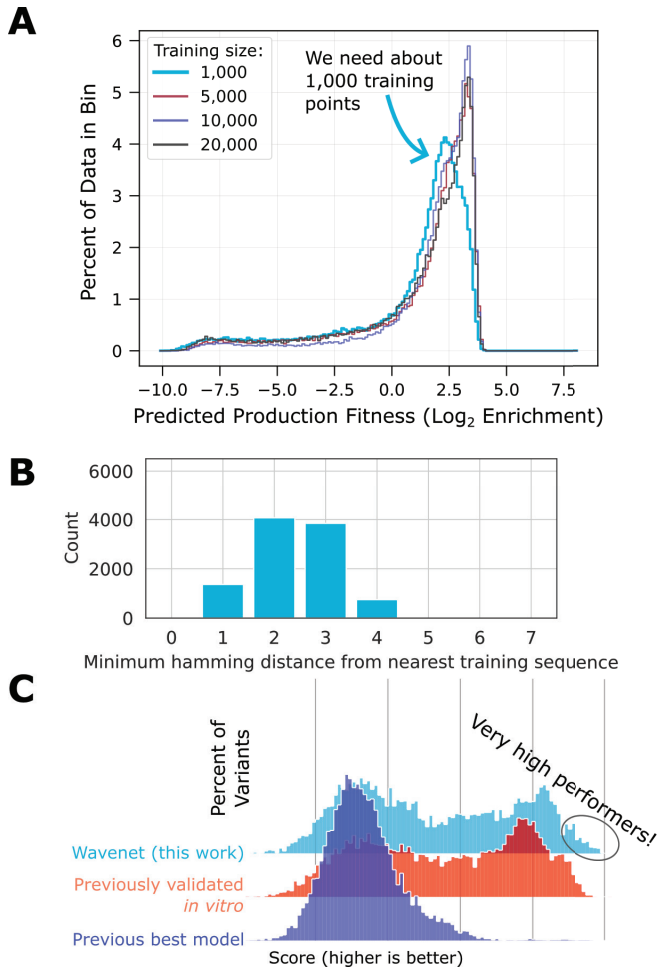

Figure 1. Wavenet model predicts high performing capsids given top examples from in vitro screens. (A) We trained Wavenet to generate production-fit variants with various amounts of training data and used

an existing model[2] to predict the production fitness of the output. **(B)** Sequence novelty as measured by the minimum hamming distance between Wavenet's output and the training set. **(C)** Wavenet was trained on top receptor targeted examples and its outputs were manufactured and tested for their ability to bind the target protein in vitro.

44 Development of Stabilized AAV Capsids for Vector Engineering

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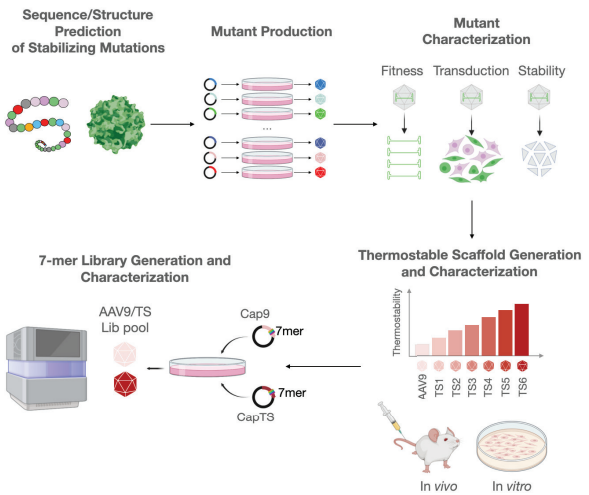
Adeno-associated virus (AAV) vectors are promising candidates for gene therapy, as illustrated by the recent FDA or EMA approvals of Luxturna, Zolgensma and Valoctocogene Roxaparvovec. One challenge in AAV capsid engineering is to enhance tropism and antibody evasion characteristics without compromising VP protein folding, capsid assembly, genome packaging and other key biological functions. Indeed, we find that loop VIII insertions, which have been used to enhance blood-brain barrier (BBB) crossing, are typically highly destabilizing. We hypothesize that AAV capsid evolvability is constrained by its stability, and that the mutational tolerance of the AAV capsid can be increased via the introduction of stabilizing, compensatory mutations. We identified and individually tested seven stabilizing mutations, which increased the AAV9 capsid melting temperature (Tm) by 1-7°C, without any major impact on production fitness or in vitro transduction phenotypes. We demonstrated that these mutations could be combined to fine tune the AAV9 capsid thermal stability (Tm = 77°C), reaching melting temperatures between 77-93.4°C without compromising production titers, in vitro transduction, or the ability to cross the BBB after systemic administration in mice. To determine whether AAV9 capsids bearing a combination of these mutations had increased mutational tolerance, we generated peptide-modified capsid libraries within AAV9 or a highly stabilized capsid variant and screened these libraries for production fitness. This work informs the development of novel AAV capsids with increased mutational tolerance for AAV engineering.



45 *In Vivo* Targeting of HSCs by Capsid-Engineered AAV Vectors

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In vivo targeting of hematopoietic stem cells (HSCs) by adeno-associated viral (AAV) vectors would open up a number of relevant therapeutic options. We therefore performed a high-throughput AAV peptide display library screen in non-human primates (NHP) for variants targeting CD34+ cells. A set of capsid variants recovered from the isolated NHP CD34+ cells was investigated as barcoded (BC) eGFP vectors in CD34+ humanized NSG mice (i.v., n = 6-7/pool, 5 d). While FACS analysis detected moderate GFP signals in PBMCs and bone marrow (BM)-derived CD34+ cells, NGS analysis of mRNA BCs identified various capsids with dominant mRNA expression in hCD34+ cells (% of total BC reads: 20-64 %). In line, several variants mediated superior ex vivo transduction of hCD34+ cells (eGFP+ cells 70-80 %). To study possible mechanisms behind the improved tropism, the above-mentioned CD34+-selective capsids were compared for their uncoating efficiency in a BC.eGFP vector pool in hCD34+ NSG mice (n= 6-7, 5 d) with AAV6 and AAV2 as controls. Here, NGS analysis focused on uncoated vector genomes in CD34+ cells, PBMCs, and liver. Thus, before NGS, isolated genomic DNA was subjected to T5 exonuclease treatment to deplete linear vector DNA (encapsidated) and rescue circular concatemeric vector DNA (uncoated). Substantial differences between candidates were observed leading to identification of two capsids, Var24 and Var30, for further analysis. In an ongoing NHP study for efficient CD34+-targeting, Var24 and 30 are compared to AAV2 and AAV6 as BC.eGFP vectors. Furthermore, relevant capsid features of Var24 and 30, such as stability, 3D structure, and immune escape (Var30), were confirmed to be altered compared to parental serotype, AAV2, and may contribute to improved CD34+ cell targeting. In conclusion, we successfully performed a CD34+ cell-targeted AAV library screen for AAV2 capsid variants in an NHP model and confirmed the species-independent tropism of several promising capsids for hCD34+ cells in humanized NSG mice and ex vivo. A number of capsid candidates is worth further investigation. In particular, Var30, with its highly efficient and specific in vivo tropism for hCD34+ cells, represents a strong candidate to equip vectors for gene therapeutic delivery to HSCs.

AAV Vectors - Product Development Manufacturing: Upstream

46 Development of a Novel Plug-and-Play Upstream Process Offering Scalable, High-Yield Production of High-Quality AAV Vectors

Klaudia Szymczak, Marissa Stanvick, Richard Gilmore, Eli Wiberg, Divya Tsiros, Shannon Wallace, Chase Rebidue, Jin Yin

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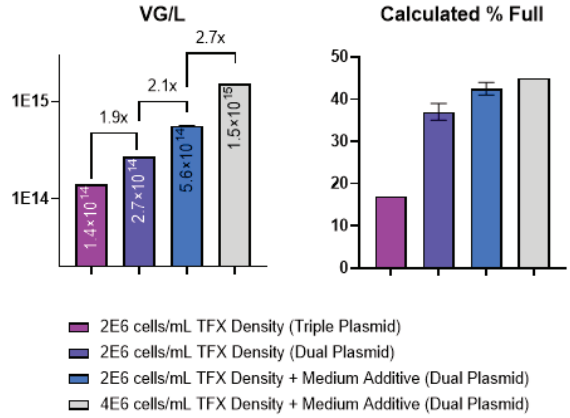
With accelerating development of novel AAV-based drug candidates the demand for high vector yields and quality has increased substantially. Improving productivity of AAV vector is critical not only to deliver adequate amounts of therapeutics to treat patients but also to bring down the cost of manufacturing per individual. To address those challenges, OXB Solutions has established a new upstream process which results in over 10-fold higher bioreactor titers with 2-fold higher percent of full capsids, and yields more than 1E15 vector genomes (vg) per L . Importantly, such productivity increase allows for coverage of 10 times more patients with ~90% decrease in production cost per individual. This significant gain in AAV titers was achieved through a combination of process changes including implementation of our novel dual plasmid system, increasing transfection cell density along with optimization of plasmid quantities, adopting a post-transfection additive, and improving bioreactor parameters (see Figure). Replacing triple plasmid constructs with the new dual configuration resulted in up to a 2-fold higher productivity and was responsible for the observed doubling of the calculated percent of full capsids. Changing transfection cell density from 2E6 to 4E6 cells/mL along with optimization of the plasmid amount per cell resulted in up to 3-fold greater AAV titers. After optimizing the final concentration and timing of addition, the additive also significantly improved the efficiency of our process with up to a 3-fold gain in generated vectors. Finally, screening of bioreactor parameters using a DoE approach identified an ideal pH setpoint which was associated with additional titer improvement. Importantly, all the increases caused by the introduced process modifications were confirmed through downstream steps and no impact on product quality was observed except for the substantial gain in percent of full capsids. In addition to high productivity, our process also demonstrates strong reproducibility among at least 9 different AAV serotypes (clades A, B, E and F) reaching bioreactor titers close to or above 1E15 vg/L, and in most cases achieving over 50% of full capsids in affinity-purified product. This reproducibility was also confirmed among various genes of interest (GOI) including GFP, luciferase, and 7 other disease-targeting genes. Finally, we successfully scaled the new process from 2L to 50L and 500L vessels, all of which demonstrated comparable titers and consistent product quality with >90% full capsids in anion-exchange chromatography (AEX)-purified product plus low levels of plasmid- and host-cell-derived impurities. Altogether, a combination of our high-performing upstream process with efficient downstream methods creates a powerful platform for AAV manufacturing yielding over 1E17 total vector genomes in the final drug substance formulation from only one 500L bioreactor. As an innovation-driven company, OXB Solutions continues to improve its platform and is currently developing a next-generation upstream process utilizing perfusion to grow and transfect cells at even higher densities. Our preliminary studies at 2L scale consistently show that this cutting-edge strategy leads to a notable increase in productivity with vg titers over 2.5E15 vg/L.



47 Analysis of Mechanisms Driving Improved Adeno-Associated Vector Production by the Addition of Small Molecules

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With the recent successes in recombinant adeno-associated virus (rAAV) mediated gene therapy, there is strong interest within the field for production process improvements to drive down costs and improve quality. Here, we take a mechanistic approach to understand the transcriptomic underpinnings of production yield using imidazole-based small molecules, which we discovered impacted production efficiency during the packaging process. To understand the baseline transcriptomic dynamics during vector production, and the subsequent changes upon small molecule addition, we used the triple transfection method to package AAV-GFP and collected total mRNA across multiple timepoints for untreated and treated samples. Using Illumina mRNA-seq, we then analyzed transcriptomic changes through time and across treatment groups. Our baseline RNA-seq results revealed significant, time-dependent changes in host cell gene expression, providing insight into expression dynamics associated with early vs late stages of vector production. We identified hundreds of differentially expressed genes after 24 hours of transfection for both small molecules, and thousands of differentially expressed genes 48 hours after transfection. Further, small molecule addition resulted in large-scale alterations to the baseline host cell transcriptome, revealing many critical gene pathways that likely underlie production efficiency, including many signaling pathways. Collectively, our results characterize gene expression dynamics operating during distinct phases of vector production and highlight pathways likely underlying production and proper generation of full and functional rAAV molecules.

48 A Modified Triple Transfection Method Produces High-Quality AAV Vectors with Much Reduced Plasmid Demand

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Triple transfection of HEK293 cells is the most widely used method for producing recombinant adeno-associated virus (rAAV). It involves three plasmids co-transfected to cells at roughly equal molar ratio: a helper plasmid that delivers certain genes of adenovirus (pAd-Helper), a trans plasmid that expresses AAV rep and cap genes (pTrans), and a cis plasmid that harbors a therapeutic transgene cassette flanked by AAV inverted terminal repeats (pCis). Although triple transfection has gained tremendous success, emerging issues arise when it is increasingly used to produce clinical-grade rAAV and scrutinized by advanced technologies, such as high cost of goods for GMP plasmid and alarming levels of plasmid backbone DNA detected in purified rAAV. Here, we describe a modified triple transfection method that improves the quantity and quality of rAAV batch with a greatly reduced demand of plasmid. We recently combined AAV-based gene delivery and suppressor tRNA-mediated stop codon readthrough to develop AAV-NoSTOP (Wang J., et al. *Nature* 2022), an in vivo gene therapy approach that targets pathogenic nonsense mutations. As we attempted to expand the AAV-NoSTOP toolbox, we failed to package certain suppressor tRNA genes into rAAV by standard triple transfection. Mechanistically, we found that expression of the suppressor tRNAs from pCis in HEK293 cells interfered with rep/cap expression from pTrans. Surprisingly, reducing the amount of transfected pCis to 1% allowed for rep/cap expression and afforded successful rAAV production. These results prompted us to carry out a series of comparative studies, which demonstrated that reducing pCis to 1% of that used in standard triple transfection was broadly applicable to both single-stranded and self-complementary vector genomes, various reporter transgenes and capsids, and different production scales. The resulting rAAV yield was comparable or slightly reduced compared with that obtained from standard triple transfection. Besides using much less plasmid, another advantage of using 1% pCis is the improvement of vector DNA purity. Specifically, PacBio sequencing showed that it significantly reduced read-through and reverse-packaged vector DNA that contained the pCis plasmid backbone (from 4.4% in standard triple transfection to 0.7%), which may cause undesired RNA expression and innate immunity in transduced tissues. Finally, in vivo studies in mice showed that rAAV produced using 1% pCis had superior transduction efficiency in a transgene-, serotype- and tissue-specific manner. In summary, we developed a modified triple transfection method that is easy to implement, cost-effective, and able to produce rAAV of high purity and potency. (#Co-corresponding authors)

49 Adenovirus L4 22 and 33K Proteins Are Essential for Episomal Amplification of Integrated rep/cap Genes and rAAV Production from Stable AAV Packaging Cells

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Recombinant adeno-associated virus (rAAV) is the vector of choice for in vivo gene therapy, however, efficient production of rAAV at scale remains challenging. rAAV packaging and producer cell lines have been explored extensively and are promising approaches for scalable rAAV manufacture. rAAV production from these cell lines is induced by infection with wild-type adenoviruses to promote amplification of integrated rep-cap genes (100 to 1000-fold), essential to supply sufficient Rep and Cap proteins for rAAV replication. This amplification effect is dependent on a cis-acting replication element (CARE). Adenovirus proteins that play a role in mobilizing CARE-dependent amplification of AAV rep-cap remain to be fully defined. We recently described a new helper adenovirus entitled TESSA, wherein the Major Late Promoter (MLP) was modified *in situ* to enable promoter self-repression for contaminant-free manufacture of rAAV. TESSA provided efficient helper functions to enable rAAV replication when the rep-cap genes were provided *in trans* but, interestingly, was unable to support rep-cap amplification and rAAV production from stable packaging cells. Here we show that expression of the adenovirus L4 22-33K unit is essential for rep-cap amplification and rAAV production using an rAAV packaging cell line HeLaRC32. Our results suggest a model wherein, normally, high levels of 22-33K are expressed from adenovirus MLP to support the replication of adenovirus and amplification of AAV rep-cap genes in the packaging cells. However, repression of the MLP from TESSA, and titration by replicating adenoviral genomes, restricted sufficient 22-33K proteins required to support rep-cap amplification. Importantly, siRNA-knockdown of the adenovirus DNA-polymerase or the use of a temperature-sensitive DNA-polymerase mutant in TESSA (TESSA-E1-tsDNA) significantly decreased adenovirus genome replication, thereby recovering rep-cap amplification for efficient rAAV replication. Our results further elucidate the intricate kinetics between CARE-dependent rAAV replication and adenoviral helper functions with implications for improving rAAV manufacture from stable cell lines.

50 A Combined Transcriptomics and Compound Screening Approach to Increasing AAV Productivity in Gene Therapy Process Development

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Biogen, Cambridge, MA

Viral vectors such as adeno-associated virus are the choice of gene delivery vehicle for Gene Therapy applications, and transient transfection using suspension cell culture remains the most widely used production platform in the industry. There is interest in continuously improving AAV productivity during the production process to meet high clinical and commercial product demands, as well as advancing fundamental understandings of AAV production

biology. Both transfection and AAV production may induce cellular stress responses including anti-viral response, which may result in reduced AAV productivity. Media additives targeting specific pathways are hypothesized to offset these cellular stress responses, and thus improve AAV production. In this work we have used transcriptomics to separate and understand the time-course cellular stress responses to both transfection and AAV production and screened small molecule compounds targeting the identified pathways. The supplementation of these compounds in production medium was further optimized using high-throughput 24-deep well and Ambr15 screening formats and confirmed in lab scale bioreactors. This combined approach of transcriptomics followed by targeted compound screening resulted in a greater than two-fold improvement in AAV productivity.

51 Development of AAV Continuous High Production Technology by FF Original Flow Electroporation Method

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The purpose of this study is to develop a technology for high production of AAV based on the triple transfection method using HEK293 cell. At present, a high dose is required in hemophilia etc., but it is difficult in principle to respond to high production with the PEI-TT method. Therefore, we are working on a technology that can achieve both high productivity and high quality by adopting a PEI-free production method that combines perfusion culture and FF original continuous flow-type electroporation technology. In this report, we will report the verification results of the technology to obtain 10L culture medium with a AAV titer of 1E+15vg/l by working on continuous production at 1L culture scale for 1 month.

52 Synthetic Cell Lines for Recombinant AAV Production

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Recombinant adeno-associated virus (rAAV) is one of the most commonly used vehicles for gene therapy due to its effectiveness in *in vivo* gene delivery, long-term persistency, and safety. In general, a very large dosage of rAAV is required in clinical applications, hence manufacturing is anticipated to be carried out in large scale to meet the demand. Current rAAV production relies on multiple plasmid transfections or helper virus infections, which pose challenge in large-scale manufacturing. Additionally, rAAV produced in current manufacturing processes often has large fractions of empty viral particles without the payload. Innovations in viral vector manufacturing are called for to increase vector titers and enhance vector quality. Our aim is to bring into being a cell line which has all the essential genetic elements for the synthesis of rAAV vectors that can be inducibly expressed to generate the recombinant virus. Since the production does not require any plasmid transfection or virus infection, the process is simple and scalable. Additionally, through tuning of the inducer concentration profile, we can control the expression dynamics of different viral components and modulate the full-to-empty particle ratio of the vector to enhance the quality of the product. We have taken a synthetic biology approach to establish rAAV producing cell lines by replacing native transcriptional regulation of viral genes with inducible promoters, organizing the essential viral genes into genetic modules, and integrating those modules independently into HEK293 cell genome. The genome module contained a rAAV genome encoding GFP; the replication module encodes the Rep68 protein and helper proteins of adenovirus; and the packaging module provided capsid proteins and the Rep52 protein. After transfection, cells with these three modules integrated into host genome were screened for rAAV productivity. To facilitate screening, we also developed an assay cell line harboring *rep* and helper genes but has no viral genome nor *cap* gene for titration of infectious rAAV. We successfully isolated four producers that can generate infectious rAAV with high titers upon induction. By optimizing the inducer ratio and induction timing, better titers with high full particle content were achieved. Transcriptomics and targeted proteomics were adopted to quantify both cellular and viral transcripts and proteins. Time-course profiles of viral transcripts and proteins revealed different viral gene expression dynamics of each cell line, and their possible influence on rAAV titers and quality. These understandings led to further modification of cultural conditions to enhance the productivity. Taken together, utilizing a systems design and synthetic biology approach, a scalable and controllable rAAV production system was demonstrated. This work exemplifies the crucial role of synthetic biology to engineer next-generation gene therapy manufacturing platforms.

Immunotherapy, Oncolytic Viruses, and Cytokines

53 UB-VV200 is a Novel Surface-Engineered Lentiviral Product Candidate for *In Vivo* Engineering of Universal TagCAR T Cells for the Treatment of Solid Tumors

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Despite the promising clinical efficacy of CAR T cells in hematologic malignancies, tumor heterogeneity and the immunosuppressive tumor microenvironment (TME) are key challenges that limit efficacy of CAR T cells in solid tumors. Additionally, complex ex vivo CAR T manufacturing and the need for lymphodepletion limit its widespread application. To overcome these challenges, our integrated platform includes a surface-engineered lentiviral vector drug product, UB-VV200, designed to specifically engineer T cells in vivo to express a universal anti-fluorescein CAR (TagCAR) and a synthetic cytokine receptor for selective small molecule- controlled in vivo expansion of TagCAR T cells. TagCAR T cells are designed

to be used in combination with TumorTags, which are bispecific adaptors comprised of fluorescein linked to a tumor-targeting ligand. UB-TT170 is a TumorTag that specifically targets folate receptors (FRα and β). FRα is upregulated on many tumor cell types while FRβ is expressed on immunosuppressive tumor-associated macrophages, which allows UB-TT170 to simultaneously target the tumor and TME. To promote selective, high avidity binding to T cells, UB-VV200 surface engineering includes an anti-CD3 single chain variable fragment (scFv) and costimulatory molecules. Here, we show that UB-VV200 binds selectively to T cells and activates them to facilitate efficient transduction in a dose-dependent manner in vitro. TagCAR T cells mediated antigen-specific cytolytic activity and cytokine release against FRα+ MDA-MB-231 tumor cells when cocultured with UB-TT170. Resulting TagCAR T cells selectively expanded through activation of the synthetic cytokine receptor. When administered in vivo, UB-VV200 efficiently generated TagCAR T cells in non-activated PBMC-humanized NSG mice bearing FRα+ MDA-MB-231 tumors. There were no acute toxicities associated with UB-VV200 administration. In vivo generated TagCAR T cells proliferated in an antigen-dependent manner in the presence of UB-TT170 and cleared established tumors. In the absence of UB-TT170, TagCAR T cells alone did not exhibit expansion or anti-tumor activity. Overall, we have demonstrated that UB-VV200 can engineer TagCAR T cells in vivo and that UB-TT170 can direct TagCAR T cells to eliminate FRα+ tumor cells. These data support development of UB-VV200 in combination with TumorTags as a new therapeutic approach against solid tumors.

54 TNT: Talimogene Laherparepvec (An Oncolytic Virus Expressing GM-CSF), Nivolumab and Trabectedin for Advanced Leiomyosarcoma: A Phase 2 Study *[NCT# 03886311]*

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Background: Intratumoral injection of Talimogene Laherparepvec (TVEC) has a local oncolytic effect and evokes a cytotoxic immune response. The combination of Trabectedin (T) and Nivolumab (N) is a safe and effective therapy in soft tissue sarcoma (STS). This study aims to determine the safety and efficacy of adding TVEC to the combination of T and N in advanced leiomyosarcoma (LMS). **Objectives: Primary:** To assess progression-free survival (PFS). **Secondary**: (1) To evaluate the best overall response, (2) PFS rate at 6 and 9 months, (3) Overall survival (OS) rate at 6, 9, and 12 months, (4) Incidence of conversion from unresectable to the resectable tumor, and (5) Incidence of treatment-related adverse events (TRAEs). **Patients and Methods:** Eligible patients included patients ≥ 18 years of age with locally advanced unresectable or metastatic LMS, measurable disease by RECIST v1.1, and at least one accessible tumor for TVEC intratumoral injection. N (3 mg/kg q2 weeks), T (1.2 mg/m2 q3 weeks), and TVEC (1x10e8 PFU/ml q 2 weeks depending on tumor size) were administered. A starting dose of TVEC (1x10e6 PFU/ml) was initially given, followed by a total dose of 1x10e8 PFU/ml q 2 weeks depending on tumor size) three weeks later. **Results: Efficacy:** Per protocol, there were 11 evaluable subjects (Modified Intention to Treat [mITT] patients who had completed at least one treatment cycle and had a follow-up CT scan). The median number of prior lines of therapy was 4 (range 1-8). Best Overall Response (BOR) by RECIST v1.1 = 2 PR, 9SD (BOR Rate 18.2%). The disease control rate (PR+SD) at week 6 was 100%. The median PFS was 7 months (range: 3- 18; Trabectedin alone for LMS= 4.3 mos; Dacarbazine alone = 1.6 mos); 6-month PFS rate, 55%; median OS 18.2 months (range: 4- 32); 6-month OS rate, 91%. Response was not related to PD-L1 positivity but both patients with PR were ER+/PR+ and had uterine LMS. There were 15 evaluable subjects for OS analysis under the Intention-to-Treat (ITT) population who received at least one dose of study drug. The median OS was 12 (range 0-32) months; 6-month OS rate, 60%. **Safety:** Eight of 15 (53.3%) patients experienced at least one ≥ Grade 3 treatment-related adverse event (TRAE). The ≥ Grade 3 TRAEs include anemia (n=3), thrombocytopenia (n=3), neutropenia (n=1), increased ALT (n=1), increased GGT (n=1), decreased LVEF (n=1), dehydration (n=1), hyponatremia (n=1). There were no new safety signals noted in this study. **Conclusion:** These results suggest that (1) By indirect comparison, the combination regimen using Talimogene laherparepvec, Nivolumab & Trabectedin may be more effective as second/third-line/fourth therapy for advanced leiomyosarcoma with manageable toxicity, (2) Response is not related to PD-L1 positivity and (3) The best responders are patients with HR+ uterine LMS.

55 Cytokine Encoding Adenoviruses Advance T cell Recruitment to Cancerous Tumors of Renal Cell Carcinoma

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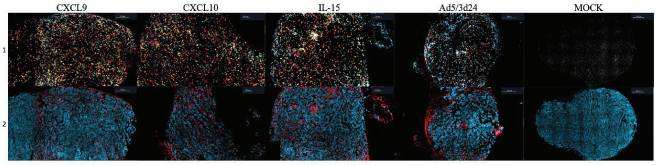
Oncolytic adenoviruses have become commonly used viral vectors for cancer immunotherapy worldwide. Such viruses have shown to work in synergy with checkpoint inhibitors, featuring a potential combination for clinical treatment. Modifications in the viral genome give further potential of such viruses to serve as gene transfer vehicles to target cancer cells of malignant phenotype, while simultaneously triggering the activity of the patient´s immune system. Traditional cancer therapies have prolonged the life of many cancer patients. However, many patients do not benefit from such therapies. This is often due to lack of tumor infiltrated immune cells. Molecular messengers known as cytokines, hold the potential to switch a cold tumor into a hot tumor with increased immune cell infiltration. Consequently, we designed cytokine encoding adenoviruses to facilitate the immune cell infiltration based on their role, mainly, in T-cell attraction. Adenoviruses encoding for CXCL9, CXCL10 and IL-15, respectively, were generated using Gibson Assembly cloning method.

To tackle the lack of relevant preclinical testing platforms for cancer immunotherapy, we use patient derived organoids from clear cell renal cell carcinoma as *ex vivo* models to predict the biological responses elicited in humans. Such patient material and T-cells matching the patient-specific immune profile from freshly isolated PBMCs, are obtained from an ongoing precision medicine study DEDUCER (Development of Diagnostics and Treatment of Urological Cancers), together with the iCAN Digital Precision Medicine Flagship. To advance the clinical relevance of our *in vitro* testing platforms, we use immunodeficient humanized NSG mice as renal cancer models to evaluate the chemotactic effect of human PBMCs *in vivo*. Here, we show the effect of oncolytic adenoviruses armed with CXCL9, CXCL10 and IL-15 cytokines to infect and secrete high amounts of respective protein in tumor cells. Consequently, immune cells are attracted and migrate to the site of infection. Furthermore, we show the presence and localization of migrated intratumoral T-cells in FFPE animal tissue samples using multiplex immunohistochemistry. An increase in the number of intratumoral CD4+ and CD8+ T-cells were observed in all groups treated with armed virus compared to unarmed and MOCK groups, suggesting enhanced immune cell -mediated cancer cell killing. The presence of transgenes was observed to be restricted to the tumor site, indicating local viral spread. In conclusion, our study show that modified adenoviruses can enhance T-cell recruitment to tumor cells both *in vitro* and *in vivo*, highlighting the potential clinical use of modified oncolytic adenoviruses as a therapeutic approach for urological solid tumors.



56 PD-L1 siRNA and Cyclic Dinucleotide Based Immune Reprogramming for Cancer Immunotherapy

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1. Introduction Immunotherapy has shown great therapeutic efficacy in blood cancer treatment, such as leukemia. However, it showed limited therapeutic efficacy in solid tumor treatment, such as melanoma and breast cancer. Several factors contribute to the low therapeutic efficacy of solid tumor treatments, including ineffective drug delivery efficacy to solid tumors, limited tumor neoantigens exposure, and an immunosuppressive tumor microenvironment. Currently, local cancer immunotherapy has attracted much attention, and many local immunotherapy clinical trials have been initiated. The underlying hypothesis is that treating locatable and accessible tumors will stimulate antitumor immunity in situ, resulting in systemic effects against distant tumors. To develop effective local immunotherapy, I have produced a nanoformulation loaded with PD-L1 and/or Cyclic dinucleotide (CDN). The nanoformulation consists of three components: an immunoative polymer 2E' that activates antigen-presenting cells to increase antigen-presenting ability; an immunogenic cell death inducer (ICD) paclitaxel (PTX) that kills tumor cells and exposes tumor neoantigens; PD-L1 siRNA (siPD-L1) inhibiting PD-L1 expression or CDN activating STING signal pathway. I have shown that a single local administration of 2E'/PTX/siPD-L1 or 2E'/PTX/CDN induces strong antitumor immunity, resulting in immediate regression of large established tumors, tumor-free survival, abscopal effect on distant tumors, and the resistance to tumor rechallenge. **2. Methods**An immunoactive polymeric assembly 2E'/PTX/siPD-L1 was developed and visualized by transmission electron microscopy. Cytotoxicity, cellular uptake, pD-L1 silencing, and immunostimulatory effects of 2E'/PTX/siPD-L1 were tested on CT26 colorectal carcinoma and bone marrow-derived dendritic cells (BMDC). Antitumor and immune activation effects of intratumorally injected 2E'/PTX/siPD-L1 and 2E'/PTX/CDN were evaluated in mouse models of syngeneic CT26 tumor and B16F10 tumor. **3. Results** *Characterization:* 2E'/PTX/siPD-L1 showed a spherical shape with an average diameter of 50 nm. 2E'/PTX/siPD-L1 showed selective toxicity to cancer cells. 2E'/PTX/siPD-L1 entered cancer cells and inhibited the PD-L1 expression in cancer cells. Furthermore, 2E'/PTX/siPD-L1 induced the secretion of immunostimulatory cytokines from immune cells and exposure of calreticulin (CRT) on cancer cells.*2E'/PTX/siPD-L1 primes systemic antitumor immunity in poorly immunogenic B16F10 tumors:* 2E'/PTX/siPD-L1 inhibited tumor growth in both treated and untreated distant tumors, yielding the smallest tumor size on both sides compared with other groups. 2E'/PTX/siPD-L1 also induced a significant increase of melanoma antigen-specific T cells compared to D5W or 2E'/PTX/siNeg (negative control siRNA), supporting that 2E'/PTX/siPD-L1 activated systemic antitumor immunity in B16F10 tumor model. *2E'/PTX/CDN eliminates established tumors and develops antitumor immunity in CT26 model:* 2E'/PTX/CDN showed the best antitumor effects: 86% of the treated mice survived tumor-free after a single treatment. When rechallenged with live CT26 cells, none of the tumor-free mice grew tumor for 66 days (duration of observation). These results indicate that the single intratumoral treatment of 2E'/PTX/CDN induced strong antitumor immune memory. However, the surviving animals did not reject unrelated syngeneic 4T1 tumors, indicating that the antitumor immunity was tumor-specific. **4. Conclusion** 2E'/PTX/siPD-L1 showed selective toxicity against cancer cells as compared to immune cells. 2E'/PTX/siPD-L1 and 2E'/PTX/CDN induced potent antitumor immunity by a single intratumoral administration, resulting in immediate regression of large established tumors, tumor-free survival, abscopal effect on distant tumors, and the resistance to tumor rechallenge.

57 Cis-Targeted Cytokines for Specific Stimulation of CAR T Cells

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INTRODUCTION In vivo behavior of CART cells correlates with improved outcomes, spurring interest in the development of approaches to selectively control their in vivo expansion after infusion. While IL-2

family cytokines can be administered to achieve this goal, cognate receptors for these cytokines exist on multiple types of immune cells, leading to toxicity and the expansion of undesired cell populations (e.g. Tregs). This problem can be addressed by engineering IL-2 variants with biased affinity to IL-2R subunits modestly increasing their selectivity, or by introducing orthogonal cytokine/cytokine receptors. The former system is not specific, and the latter is complicated by the need for genetic modification of cytokine receptors on engineered T cells. To address these challenges, we developed IL-2 or IL-21 fusion molecules that selectively activate CART cells by recognizing an extracellular tag that is an integral component of existing, commercially available CARs. METHODS Cis-targeted cytokine fusions are comprised of (1) a targeting antibody directed against an exogenous tag expressed on the CAR-T surface (truncated non-signaling epidermal growth factor receptor [EGFRt] that is co-expressed with the CAR) and (2) a cytokine mutein with attenuated binding to its cognate receptor. Specifically, IL-2 muteins exhibit diminished binding to IL2Rα/β while the IL-21 mutein has diminished binding to the IL-21R subunit. The fusion molecule targeting arm provides avidity to the attenuated cytokines, resulting in selective activation of the associated cytokine receptors on CAR-Ts. Both molecules were characterized in vitro using primary human CAR T cells. In vivo activity was tested by IV injection of ultra low-dose human CART19 (1 x 10^5) into immunodeficient mice engrafted with a B-ALL cell line. Cis-targeted cytokine fusions were administered i.p. once, one day later. Anti-tumor activity was measured by bioluminescence imaging (BLI) and analysis of peripheral blood were performed weekly to examine the expansion, persistence, and phenotype of the CAR-Ts. RESULTS The specificity of the EGFRt-IL2 molecule was demonstrated by its ability to selectively induce pSTAT5 signaling and EGFRt-IL21 molecule by pSTAT3, resulting in >100-fold preferential STAT activity in CAR-T cells compared to non-CAR cells. In vivo BLI revealed that when a suboptimal dose of CAR-Ts were injected into leukemia-bearing mice, both EGFRt-IL2 and EGFRt-IL21 induced substantial tumor regression (Figure 1). EGFRt-IL2 induced a stronger initial expansion of CART19 in vivo than EGFRt-IL21 (median 964.98 CART19 T cells/μL blood vs 22.98 CART19 T cells/μL blood, p< 0.0001 on day 13). Both therapies were superior to CART19 therapy alone without exogenous cytokine and were well tolerated in mice. CONCLUSION Cis-targeted IL-2 or IL-21 cytokine fusion molecules selectively activate CAR-Ts in vitro and enhance in vivo anti-tumor activity and survival. Temporal control of the cis-targeted cytokines directed by anti-tag antibodies represents a promising approach to enhance CAR T cell therapies.

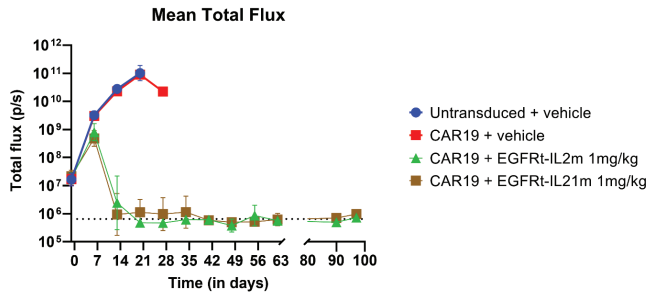

Figure 1.

58 Correlative Results of a Phase 1/2 Study of Pembrolizumab Combined with Blinatumomab in Patients with Relapsed/ Refractory (r/r) ALL

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Blinatumomab (blina), a bispecific anti-CD19/CD3 antibody, has resulted in improved outcomes in patients (pts) with r/r B-ALL compared to conventional chemotherapy. However, over half of treated pts fail to respond (CR/CRh/PR, 44%) and the majority of responders eventually relapse without consolidation therapy. Upregulation of PD-L1 on leukemic blasts and PD-1 on T cells have been shown to associate with resistance to blina in preclinical and clinical studies. Blockade of the PD-1 pathway represents a reasonable strategy to augment blina efficacy. Thus, we set out to test the combination of pembrolizumab (pembro) and blina in a single arm, phase 1/2 trial (NCT03512405). We investigated preliminary correlative results from samples collected from patient peripheral blood (PB), bone marrow (BM), and serum, including immune cell phenotypes via multi-color flow cytometry (MFC), the TCR repertoire via TCRβ sequencing, and serum cytokine levels via the Luminex Cytokine 30-Plex Human Panel. Pts (≥18 years; ECOG <1) with CD19+ r/r B-ALL received standard blina 9 μg/day on days 1-7 and 28 μg/day on days 8-28 in cycle 1 and on days 1-28 in subsequent cycles. Pembro 200 mg was given on day 15 in cycle 1 and on days 1 and 22 in subsequent cycles. PB and serum samples were collected at pretreatment and on days 1, 8, 15, 22, and 29 of cycle 1, at the end of subsequent cycles, and at standard of care (SOC) times. BM samples were collected before treatment, in the last week of cycles 1 and 2, and at SOC times. We used conventional and spectral MFC to phenotype immune cell populations in PB and BM, including T regulatory (Treg) cells, tumor cells, and T cells. We also quantified the number of CD19 molecules using quantitation beads. Additionally, we used the ImmunoSeq platform to do bulk TCRβ sequencing of PB samples at pretreatment and days 1, 8, 15, 22, and 29 after the first cycle and on day 36 after the second cycle of treatment. Finally, we measured serum cytokine levels using Luminex. As of January 6, 2023, 7 pts were enrolled to phase 1 with one unevaluable, and 12 patients to phase 2. Eighteen evaluable pts received a median of 2 (1-6) cycles of treatment. Thirteen of 18 evaluable pts (72%) achieved flow cytometry MRD negative (MRD-) CR/CRi after a median of 1 (1-2) cycle. Twelve of 18 evaluable pts were still alive at the data cut-off. Flow cytometry analysis showed that PD-1 detection decreased among both responders (n=12) and non-responders (n=5) after pembro administration in T cells from PB, but not among responders (n=12) and non-responders (n=4) in T cells from BM. There was no difference in the percentage of Treg cells in responders vs. non responders in PB and BM. Responders after cycle 1 had higher CD8+ T cells which increased further in PB but not BM following pembro administration. Additionally, we did not detect expression of exhaustion markers in non-responders. We quantitated CD19 levels in B cells at pretreatment and preliminary results suggest CD19 expression levels do not correlate with response. Serum cytokine levels suggest no

differences in levels among responders (n=9) vs. non-responders (n=4). Correlative studies, including bulk TCRβ sequencing analysis is ongoing. Encouraging safety and anti-leukemic activity have been seen in the majority of treated patients (CR/CRi, 72%), and most were MRD-. The phase 2 portion of the trial continues to recruit patients. Preliminary correlative studies suggest PD-1 levels are higher in BM vs. PB from pretreatment and day 29 of cycle 1. Additionally, Treg cells were not elevated in non-responders as has been previously described. The quantitation of CD19 in BM suggests that high CD19 levels are not a prerequisite for response.

59 Epigenetic-Mediated Cancer Rewind Enhances Oncolytic Immunovirotherapy

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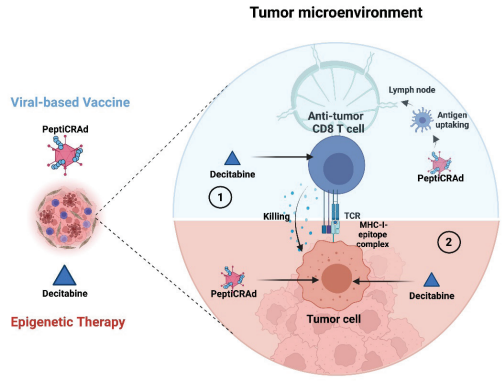
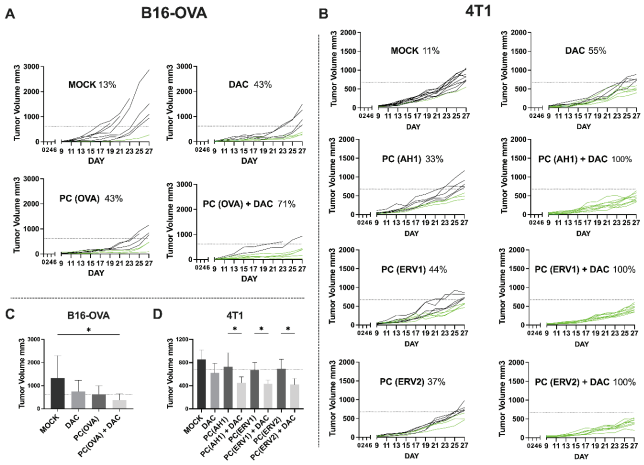
Cancer immunotherapy requires the natural or artificial generation and boosting of a specific anti-tumor CD8+ T cell-driven immune response. To be recognized and killed by effector CD8+ T cells, tumors should have functional and sustained antigen expression within the MHC-I complex of mutated or tumor-associated antigens (TMAs or TAAs); however, upon genetic and epigenetic alterations of the antigen processing and presenting components, cancer cells escape anti-tumor immune response within the tumor microenvironment (TME). As a result, poorly immunogenic tumors are refractory to conventional immunotherapy and oblige patients to severe chemotherapy and radiotherapy regimens. In this context, oncolytic viruses (OVs) and DNA methyltransferase inhibitors (HMAs) represent promising tools to boost cancer immunogenicity by enhancing cancer antigen presentation and improving CD8+ T cell effector functions in-vivo. Because of that, combining these two agents constitutes a potential synergistic strategy to overcome cancer immunotherapy limitations. However, studies applying epigenetic therapy benefits to viral-based immunotherapy are limited so far. In this study, we evaluated FDA-approved HMAs in combination with OVs for boosting tumor immunogenicity in melanoma and triple-negative breast cancer (TNBC). Specifically, we assessed the combined impact on HLA-I surface expression in cancer cell lines and activation of human CD8+ T cells. Then, we tested our approach in cancer-bearing mice where the epigenetic therapy enhanced oncolytic therapy in controlling the tumor growth, remodeling of the TME, and improved specific anti-tumor CD8+ T cell response generation.



Decitabine (DAC) promotes CD8+ T cell activation and boosts tumor immunogenicity in the tumor microenvironment (TME). Synergistically, our viral-based immunotherapy PeptiCRAd reshapes the TME and primes specific anti-tumor CD8+ T cells. The combination of the two agents generates an enhanced anti-tumor immune response.



Tumor growth in a syngeneic mouse model of B16-OVA and 4T1. Mice were treated with PBS (Mock), Decitabine (DAC), Oncolytic vaccine (PeptiCRAd, PC), and PC + DAC. Tumor growth curves for each treatment are shown in (A) and (B); the number of mice in the B16-OVA model was 7; the number of mice in the 4T1 model was 9-10. Tumor size (mm3) at day 27 post-tumor implantation in B16-OVA (C) and 4T1 (D) models. Levels of significance were set at *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (two-way ANOVA with Tukey's multiple comparisons correction to compare individual groups). Single curves represent individual mice for each treatment group. The dotted line represents the tumor volume threshold to define responder mice. Experimental group names are shown together with the relative responder percentage. Bar plot graphs are shown as mean ± SD.

AAV Vectors - Preclinical and Proof-of-Concept In Vivo Studies I

60 AAV Immuno-Gene Therapy Delivers Vectorized Cytokines to Effectively Treat High-Grade Gliomas

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High grade gliomas (HGG) are highly refractory to treatment. Innate interferons (IFN) are pleiotropic immunomodulatory cytokines central to the body's basal cancer immunosurveillance and many therapy-induced immunogenic anti-tumor responses. IFNs drive many anti-tumor functions including being anti-angiogenic, increasing tumor MHC-I expression, stimulating apoptosis, enabling DCs to mature and cross prime T cells against tumor antigens, and increasing the proliferation of activated macrophages and NK cells. Delivering gene therapies to the brain with AAV has proven to be safe and effective. However, common delivery routes (intrathecal, intracerebroventricular and intracisternal) are broadly dispersive and non-targeted. Direct intratumoral delivery limits off-target transduction and reduces neurotoxicity risks. Brain cancer patients often undergo surgical resection or biopsy, providing an opportunity to concomitantly infuse AAV directly using Convection-Enhanced Delivery (CED). This precise and highly targeted method allows for significantly reduced vector doses and improved safety. Here we evaluated safety and efficacy of an immuno-gene therapy utilizing AAV9 vectors to express engineered immunomodulatory payloads (IFNα1, IFNβ, IFNγ, and combinations thereof). We first assessed tumorigenesis and intrinsic therapeutic responses to our suite of vectorized IFNs in primary human HGG organoids. PBS, DMSO and AAV-GFP had no effect on tumor or healthy cerebral cells in the organoids, and HGG tumor cells grew uncontrolled. Temozolomide chemotherapy (the current standard of care) significantly decimated healthy cerebral cells and only slightly delayed HGG tumor growth. In contrast, our AAV immuno-gene therapies with 12 different engineered cytokine payloads, rapidly and selectively reduced tumor size. We next tested our payloads *in vivo* in 3 orthotopic HGG mouse models delivered intratumorally via CED. Our AAVs effectively blocked tumor growth, resulting in tumor regression and significantly prolonged survival in human GBM6 xenografts ($P<0.02$-0.001 and 31-60% complete responses (CR)), mouse GL261 allografts ($P<0.0009$), and human patient-derived xenografts ($P<0.04$; 30% CR); ($n = 450$). Evaluation of tumor-bearing mouse brains demonstrated marked tumor changes following treatment. Within 48hrs following treatment, local intratumoral expression of our engineered IFNs led to widespread tumor cell apoptosis, activated microglia, and reactive astrogliosis. Remarkably, by day 7, there was reproducible tumor eradication, no evidence of residual proliferating cells, no engineered cytokine payload expression, and only residual reactive astrogliosis and microglial responses. This was seen at both the protein level by immunohistochemistry, and at the transcript level by RNAscope. To further probe transcriptomic responses, we performed single cell sequencing on syngeneic mouse brain tumors following treatment and demonstrated that tumor cells specifically exhibited a significant upregulation of genes linked to IFN responses (*Ifit1, Ifit2, Ifitm3*), as well as other immune response genes (*Isg15, Bst2, Trim30a*) and were specific to IFN payload expression, not AAV. These transformative results introduce universal AAV immuno-gene therapies as a promising new drug class that we're advancing to the clinic to treat solid tumors.

61 Durability of Bicistronic AAV Gene Therapy for Tay-Sachs Disease in the Sheep Model

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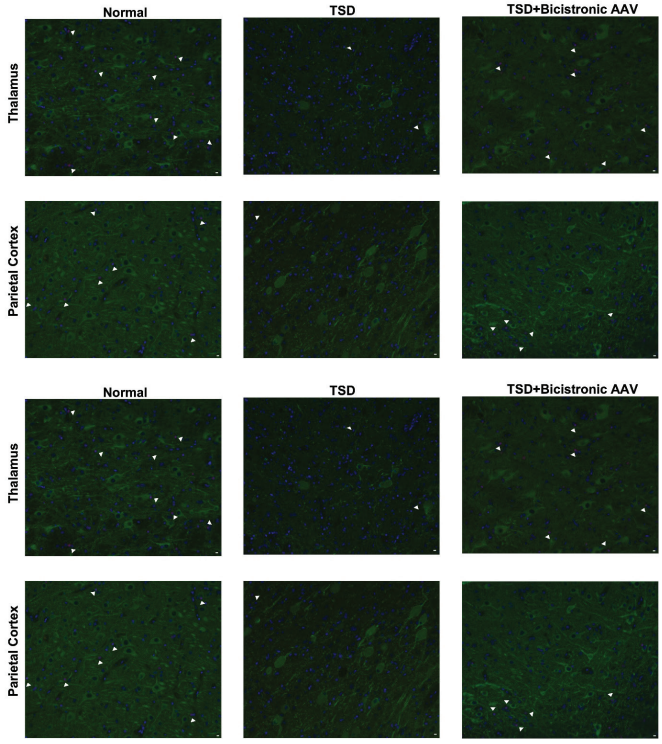
Tay-Sachs and Sandhoff diseases (TSD, SD) are fatal neurodegenerative disorders, caused by mutations in alpha or beta subunit respectively, of enzyme Hexosaminidase A (HexA). Hex A deficiency results in GM2 ganglioside storage and subsequent neuronal death. While infantile and juvenile forms are fatal, late onset patients become wheelchair bound in mid-life.Here we update on preclinical efficacy of a bicistronic AAV9 gene therapy in TSD sheep after combination of bilateral ICV, cisterna magna, and lumbar intrathecal cerebrospinal fluid injections (CSF, n=5 long term; n= 4 at 5 months). Survival is currently at 41±18 months, with two sheep ongoing. Neurologic disease was mild at euthanasia for all the deceased sheep who were euthanized due to 1) cervical trauma from ramming at 10 months 2) right sided heart failure at 46 months and 3) metacarpal ligament rupture at 42 months. The remaining two sheep presently have mild neurological disease at 48 and 60 months. The untreated TSD sheep lifespan is ~ 9±0.5 months. Post-mortem: The CSF treatment cohort resulted in above normal Hex A levels (3X-20X) throughout the brain and spinal cord at 4 months and 4 years after gene therapy. GM2 levels in brain and spinal cord returned to normal, except for thalamus and temporal cortex where GM2 levels decreased by 50%. Histopathology in TSD brain showed reduced number and swollen neurons (MAP2 staining) and a reduction of oligodendrocytes (olig2). In CSF AAV9 treated sheep, normalized neuronal morphology and increased number of oligodendrocytes (Fig. 1) was observed. Staining for astrocytes (GFAP) and glia (Iba1) is ongoing, and preliminarily a reduction of gliosis is observed in the in CSF treated cohort. Biomarkers: HexA in CSF of long-term cohort remained in normal range up to 3 years, after which a slow but steady decrease was observed. The inverse was observed where with GM2 in CSF. Interestingly, the CSF treated cohort had serum

anti AAV9 neutralizing antibodies (Nab)>1:10 at time of treatment that increased over period of 4 months for three sheep. Two sheep that showed highest pretreatment titer at 1 and 2 months, had 50% less HexA in CSF. Magnetic resonance spectroscopy and diffusion tensor imaging showed normalization of markers of neuronal health, myelination, microstructural integrity and metabolism. We are moving toward a human clinical trial for juvenile and late onset TSD and SD patients using combined bilateral ICV, cisterna magna and lumbar intrathecal AAV9 injection (1.5E15vg divided across sites). The pre-IND application was agreed upon by FDA and includes a non-GMP AAV in phase I/II clinical trial, a GLP-like non-human primate study (n=6) and a full GLP rat study.**Fig 1**. MAP2 (green) and Olig2 (magenta, arrowhead) IF in thalamus and parietal cortex of normal, untreated TSD sheep and bicistronic AAV treated TSD sheep. Scale bar 5µm.



62 More Than Nine Year Survival of a GRMD Dog after Injection of AAV-Microdystrophin Gene Therapy

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Duchenne Muscular Dystrophy (DMD) is a X-linked inherited muscle-wasting disease primarily affecting young boys with a prevalence of 1:5,000. The disease is caused by loss-of-function mutations in the gene encoding for the Dystrophin protein and is characterized by systemic, progressive, irreversible and severe loss of muscle function. Among vector systems that allow efficient *in vivo* gene transfer, recombinant Adeno-Associated Virus vectors (rAAV) hold great promise and allow very efficient transduction of skeletal and cardiac muscles. However, full-length dystrophin cDNA exceeds the packaging capacity for a single rAAV gene-delivery cassette. Therefore, truncated versions namely microdystrophins (MD) have been designed and optimized to contain few clinically important regions of the dystrophin protein. In particular, a MD variant, termed MD1, was optimized for mRNA stability and translation efficiency, and packaged in a rAAV8 under the control of a synthetic, muscle- and cardiac-restricted promoter. We previously demonstrated safety and 2-year efficacy of rAAV8-cMD1 (canine MD1) gene therapy after systemic peripheral administration of vector in Golden Retriever Muscular Dystrophy (GRMD) dogs, the canine model of DMD. GRMD dogs received at 2 months of age a systemic intravenous administration of 1E14 vg/kg of rAAV8-cMD1 without immunosuppression. This results in significant and sustained levels of microdystrophin in skeletal muscles and reduces dystrophic symptoms. No toxicity or adverse immune consequences of vector administration were observed for over 2 years after rAAV-MD injection (Le Guiner et al, 2017). This study, among others, paved the way towards a phase 1/2 multicentric clinical trial of single administration by intravenous (IV) route in 6 to 10-year-old ambulant boys with DMD, that was initiated in 2021 by Genethon. Most of the GRMD dogs that were included in our preclinical study were sacrificed few months after injection in order to document the efficacy and the safety of the treatment. However, we maintained one dog for very long-term follow-up. As of today, this dog is still alive, more than 9 years post-vector injection, whereas life expectancy of untreated GRMD dogs rarely exceeds 2 to 3 years. During these 9 years, this very active dog showed stable clinical scores, between 70 and 80 % (a score of 100% being those of healthy golden retriever dogs). Gait improvement, evaluated using Locometrix system, was also preserved during these 9 years, at levels that were very close to healthy dogs scores. All clinical parameters, including cardiac function, respiratory function, digestive function, neurological function, but also blood clinical chemistry and hematology parameters showed no or only minor evolution during this follow-up. This striking preservation of the cardiac function is never seen in our untreated GRMD dogs. No deleterious immune response (against cMD1 or AAV8) was detected either. All the data obtained during this multi-year follow-up will be presented. At a time when several rAAV-MD products are being tested in young DMD patients, such results are of importance to demonstrate that this gene therapy can be sustained for many years, with no decline in clinical improvement.

Reference: Le Guiner C et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. Nat Commun. 2017;8:16105.

63 Intravitreal Gene Therapy of Retinitis Pigmentosa (RP) Associated with Mutations in the *CNGA1* Gene (*CNGA1*-RP)

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Pathogenic sequence variants in the cyclic nucleotide-gated (CNG) channel subunit alpha 1 (*CNGA1*) gene cause autosomal recessive retinopathy of the retinitis pigmentosa (RP) type. Treatments for *CNGA1*-RP are currently not available. In this study, we developed and tested a novel intravitreal gene supplementation therapy suitable for clinical translation. The therapy uses a novel recombinant adeno-associated virus (AAV) vector based on the engineered AAV2.NN capsid, carrying a genome that features a human rhodopsin promoter driving rod-specific expression of full-length human CNGA1. AAV2.NN.hCNGA1 was evaluated for potency and safety *in vitro* and *in vivo*. In particular, *in vitro* transgene expression was confirmed at the transcript and protein level in HeLa and HEK293 cells engineered to transactivate expression from the rhodopsin promoter. In addition, transgene expression was established in human retinal organoids grown from hIPSCs. *In vivo* potency testing was performed in the *Cnga1* mutant (*Cnga1*MUT) mouse model of *CNGA1*-RP that lacks expression of the rod CNG channel. Increasing doses of AAV2.NN.hCNGA1 were delivered through single intravitreal injection in 3-week-old *Cnga1*MUT mice and the treatment effect was assessed over a follow-up period of 8 weeks at the level of retinal morphology, retinal function, and transgene expression. We found that intravitreal treatment with AAV2.NN-CNGA1 resulted in efficient expression of the human CNGA1 protein in mouse rods and was able to normalize the expression of the endogenous mouse CNGB1 subunit, which together with CNGA1 forms the native heterotetrameric CNG channel in rod photoreceptors. The treatment led to a dose-dependent recovery of rod photoreceptor-driven function and preservation of retinal morphology in *Cnga1*MUT mice. A GLP-safety study in rabbits applying single intravitreal injection followed by a 6-month post treatment observation period confirmed safety of AAV2.NN.hCNGA1. In summary, these results demonstrate the efficacy and safety of hCNGA1 gene supplementation therapy and support the translation of this approach toward future clinical application.

64 AAV Vaccine in a Preclinical Spontaneous Canine Model of Oral Melanoma

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Oral melanoma (OM) is a disease with low incidence but high mortality in the US. Early detection is difficult, so metastasis in distant organs is the main cause of death. Standard of care therapy involves intense chemotherapy and radiotherapy, which may cause severe adverse events and it is generally ineffective. In contrast, immunotherapies including cancer vaccines, harness of the immune system of the patients to control and eliminate malignancy, sparing healthy cells and tissues. Similar to mucosal melanoma in humans, canine OM is a very aggressive disease that readily spread to other parts of the body particularly the lungs and lymph nodes with a life expectancy of 6-12 months with standard of care treatments. Like humans, dogs with naturally occurring tumors have an intact immune system and many studies have shown that dogs offer a robust, clinically relevant model to study novel immunotherapies. In current pilot study, we used novel AAV-based vaccination in dogs with spontaneous OM in conjunction with standard of care local therapy. The novel vaccine developed in our lab addressed several limitations of previous AAV vaccines, such as low efficiency and immunogenicity, lack of ability to activate $CD4^+$ T helper cells, and functional impairment of $CD8^+$ T cells caused by persistence of antigen expression at the site of injection. The major innovation of the vaccine is fusion of truncated tumor antigen with MHC class I molecule-trafficking signals, which dramatically increases antigen-specific $CD4^+$ and $CD8^+$ T cell expansion and their killing capabilities. Specifically for dog study we used optimized AAV-based multivalent vaccine carrying pre-melanosome protein gp100, Tyrosinase (Tyr) and tyrosine-related protein 1 (TRP-1) to overcome tumors heterogeneity and their ability to escape immune control by losing expression of targeted antigens. Serum was collected from dogs prior to enrollment for evaluation of anti-AAV neutralizing antibody assays (NAB) and to elucidate the suitability of the candidates for the vaccine treatment. The threshold was set at NAB titter ≤1:8. Four (4) dogs were already enrolled in pilot study with major goal to establish toxicity-less minimal dose at $1x10^{12}$ vg/per dog containing equal load of each of the three tumor antigens. The parameters of the study to determine the success of the treatment were collected at different time points include physical evaluation at the veterinary clinic to assess the general welfare of the dogs, blood biochemistry and hematological analysis. Chest radiographies were taken before and after surgery to follow up for lung metastasis. The peripheral blood mononuclear cells (PBMCs) were collected for immunological assessment of antigen-specific $CD8^+$ T cells by IFN-γ ELISPOT assays. We have documented several important observations. None of the dogs experienced adverse events related to the vaccine. One out of four dogs showed clear evidence of immune response to all three AAV encoded antigens. One dog experienced local recurrence and metastasis 3 months after screening and the other dog displayed pulmonary metastasis 6 months

after screening. The other two dogs are alive at the time of writing with no evidence of recurrence or metastasis. Our study documented for the first time the safety of AAV expression tumor antigens in dogs with spontaneous cancer and general utility of non-mutated shared tumor antigens as targets for cancer vaccination.

65 The Longitudinal Kinetics of AAV5 Vector Integration Profiles in Mice

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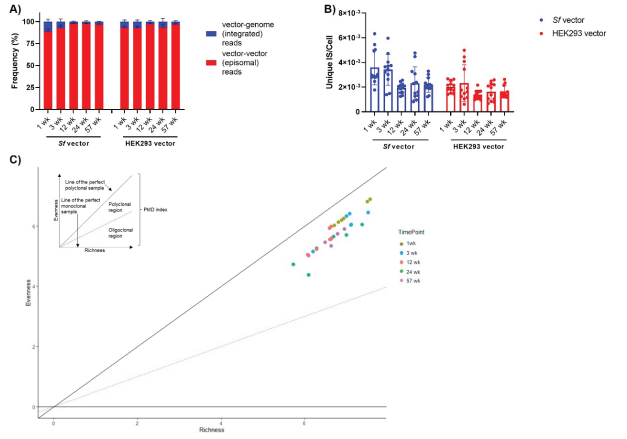
Introduction: Recombinant adeno-associated virus (rAAV)-based vectors are used clinically for gene transfer for their ability to effectively transduce human cells. Most rAAV vector genomes persist in cells as extrachromosomal episomes. Vector genomes can integrate into the host genome, but the theoretical risk of tumorigenesis may depend on vector regulatory features. We use a mouse model to investigate the long-term kinetics and integration profiles of an rAAV serotype 5 (rAAV5) vector that mimics key features of valoctocogene roxaparvovec (AAV5-hFVIII-SQ), a gene therapy for severe hemophilia A. **Methods:** We used an rAAV5-human alpha-1 anti-trypsin (hA1AT) vector approximately the same size (~5 kb) and containing the same regulatory elements as valoctocogene roxaparvovec. Mice do not develop antibodies to hA1AT protein, thereby allowing long-term studies. C57BL/6J mice received $6x10^{13}$ vg/kg of vector produced in insect *Spodoptera frugiperda* (*Sf*) and HEK293 cells. Mice were sacrificed at weeks 1, 3, 12, 24, and 57, and DNA and RNA extracted from liver samples. Target enrichment sequencing, common integration site (CIS) analysis, and polyclonal-monoclonal distance (PMD) tools were used to characterize vector integration profiles over time. **Results:** The majority (88.7%-97.4%) of vector genome reads retrieved from *Sf* vector-treated mice contained vector-vector junctions, with high representation of inverted terminal repeats, representing the episomal form of the vector (**Figure 1A**). The average vector integration frequency was 2.68 (SD, 0.77) integrations per 1000 cells, and remained constant up to 57 weeks post-dose (**Figure 1B**). To determine if integration sites (IS) accumulate at specific genomic regions, CIS analysis was performed, which attributes a CIS order based on the number of unique integrations spanning a CIS region. In samples from *Sf* vector-treated mice, 5.46% (357 of 6533 total) of CIS had an order ≥5, suggesting a proportion of these integrations occurred in a non-random manner. Integrations were enriched near the transcription start sites of genes highly expressed in the liver ($P = 1x10^{-4}$), and less enriched for groups of genes with low or no liver expression. We used a PMD index tool to evaluate the diversity of IS across samples. This tool estimates clonality by measuring distance between richness, represented by the number of IS within a sample, and evenness, represented by the relative frequencies of each IS. The *Sf* vector-treated samples clustered near the theoretical maximum for polyclonality, indicating a lack of clonal expansion throughout the study (**Figure 1C**). This provides molecular support for the absence of tumors observed by histology in vector-treated mice. Results from mice treated with HEK293-produced vector confirmed those from *Sf*-produced vector. **Conclusions:** Our longitudinal integration analysis suggests the AAV5-hFVIII-SQ integrations occur within 1 week at a low frequency and do not increase with time. Most importantly, we found no evidence of clonal expansion at a molecular or histological level.

Figure 1. Dynamics of vector integration. **A)** Frequency of episomal vs genome integrations in vector-treated mice. **B)** Unique IS frequencies. **C)** PMD clonal framework.



Based on the properties of Rényi entropies (Rényi, 1961), a clonality plane was constructed considering two extreme components of diversity: richness (total number of IS) and evenness (abundance of each IS). Distance from theoretical maximal polyclonality and monoclonality defines the PMD.
HEK293, human embryonic kidney 293 cells; IS, integration site; PMD, polyclonal-monoclonal distance; Sf, Spodoptera frugiperda; wk, week.

RNA Virus Vectors for In Vivo and Ex Vivo Applications

67 Macrophage Inhibitor Clodronate Enhances Liver Transduction of Lentiviral but Not AAV Vectors or mRNA Lipid Nanoparticles *In Vivo*

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Recombinant adeno-associated virus (rAAV) are leading vectors for liver-directed gene therapy. rAAV vectors have been reported with severe adverse events when targeting other organs with high doses. rAAV integration, even at low rate, holds a theoretical risk of insertional mutagenesis. Alternative liver-targeting gene therapy strategies are being developed such as integrating lentiviral (LV) vectors or lipid nanoparticles encapsulating mRNA (LNP-mRNA). For all these strategies to deliver gene therapy, safety is paramount and

administering the lowest minimal effective dose is essential to prevent potential adverse events. Macrophages act as sentinels of the immune system and prime the innate immune response. To maximise safety and increase efficacy, we investigated the potential of macrophage inhibition on liver targeting for two main liver-targeting viral delivery systems, rAAV and LV vector, and with non-viral delivery system, LNP-mRNA. Clodronate is a bisphosphonate which transiently depletes liver and spleen macrophages by 80-90%, following systemic administration. We assessed side-by-side liver transduction after systemic pre-treatment of clodronate liposomes, in both neonatal and young adult wild-type mice for the rAAV (serotype 8) and third generation LV vector modality using GFP. Neonatal and adult clodronate-treated mice showed 10-fold and 2-fold increase of LV-transduced hepatocytes respectively, assessed by immunostaining. Adult clodronate-treated mice showed over 7-fold increased lentiviral vector genome copies (VCN) per cell in the liver. Neonatal and adult mice injected with rAAV did not show any significant difference in transduction in both groups pre-treated with clodronate or not. Similarly, assessing liver GFP protein expression following administration with LNPs encapsulating GFP encoding mRNA, via western blot, did not show difference in the young adult group despite clodronate pre-treatment. This study demonstrates that only LV vector but neither rAAV vectors nor LNPs transfection significantly benefit from systemic clodronate pre-treatment to improve liver transduction. These findings will have translational application for liver-targeted gene therapy programmes.

68 Liver-Directed Lentiviral Gene Therapy is Safe and Curative in Argininosuccinic Aciduria

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The liver-based urea cycle enables nitrogen waste and clearance of neurotoxic ammonia. Argininosuccinic aciduria (ASA) caused by argininosuccinate lyase (ASL) deficiency is the second most common inherited urea cycle defect. Patients present either with neonatal- or late-onset hyperammonaemia, which causes coma and death if untreated, and a high risk of severe cognitive impairment and epilepsy. Curative liver transplantation can be performed in severe cases but requires lifetime immunosuppression. We aimed to test *in vivo* lentiviral gene therapy in neonatal ASL-deficient ($Asl^{Neo/Neo}$) mice. $Asl^{Neo/Neo}$ pups received an intravenous injection of lentiviral vector encoding codon-optimised human *ASL* (LV.coASL) at 4E10TU/kg, versus a control vector encoding GFP at the same dose. LV.coASL-injected animals survived the 12 weeks experiment whilst control mice died within 4 weeks ($p<0.001$). Growth ($p<0.01$), fur coat pattern, ammonia ($p<0.001$), plasma arginosuccinate ($p<0.001$), citrulline ($p<0.01$), and orotate ($p<0.05$) were normalised to those in wild-type mice. Significantly increased ASL expression (+300%; $p<0.01$) and activity (+30%; $p<0.05$) were observed in treated $Asl^{Neo/Neo}$ livers compared to controls. Lentiviral vectors present long-term transgene expression due to their ability to integrate in the host genome. We conducted safety studies to address biodistribution and the presence of genotoxic events driven by the lentiviral vector *in vivo*. 20 neonatal wild-type mice received LV.cohASL vector intravenously versus 20 PBS-injected littermates. No significant difference was shown for survival and liver/body weight ratio. Pathology analysis and dissection of the liver, spleen, and major organs showed no lesions, cysts, or any signs of tumours. Lentiviral biodistribution was predominant in the liver and to a significantly lower extent in the spleen and lungs. This positive outcome was further confirmed by the integration site analysis performed on murine livers at 1 and 9 months after *in vivo* transduction and in human primary hepatocytes at 72 hours after *in vitro* transduction. No clonal dominance was observed so far, and no known oncogenes were over targeted by the lentiviral vectors. Overall, our preliminary studies demonstrated proof of concept of efficacy and safety of *in vivo* lentiviral gene therapy for ASA.

69 Characterization and Cure of a New Mouse Model of alpha-Thalassemia Major

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α-Thalassemia (α-thal) is caused by insufficient production of the α-globin protein. In patients with severe α-thal, chronic blood transfusion or allogeneic bone marrow transplantation are required for survival. Alternatively, patients could be treated by autologous bone marrow transplant (BMT) following *ex vivo* transduction with a human α-globin expressing lentiviral vector. However, a proper model system and vector must be established for the development of such a treatment. We created a conditional knockout (cKO) mouse model that has been edited to remove *Hba-a2*, while inserting loxP sites flanking *Hba-a1* ($Hba\text{-}a1^{fl}/Hba\text{-}a2^{ko}$). Homozygous cKO animals ($Hba\text{-}a1^{fl/fl}/Hba\text{-}a2^{ko/ko}$) demonstrate a mild phenotype. To achieve complete deletion of the α-globin genes ($Hba\text{-}a1^{ko/ko}/Hba\text{-}a2^{ko/ko}$) in HSC we developed a novel lipid nanoparticle (LNP) preparation with an antibody to target CD117 and deliver nucleoside-modified mRNA coding for Cre recombinase. Cells showed the expected deletion of the α-globin genes and were injected into myeloablated recipient mice. These chimeras demonstrated a lethal phenotype characterized by abnormal red cells only expressing abnormal hemoglobin (β-chains tetramers/HbH), elevated hematocrit, splenomegaly, and iron deposition in the liver, as well as high levels of erythropoietin in the kidney and low levels of hepcidin in the liver (Fig. 1A). We screened multiple potential erythroid specific lentiviral vectors in both mouse and human cell lines and identified a promising candidate, ALS20αI, which at VCN=1 produced human α-globin protein equivalent to that of a single endogenous

α-globin gene. Myeloablated recipient mice transplanted with Cre-CD117-LNP-treated cKO BM die roughly 7 weeks post-transplant with the expected pathological phenotype. In contrast, mice receiving BM treated with Cre-CD117-LNP and ALS20αI (n=10 and VCN>1) all survived long term (>5-months) with normalization of erythropoiesis and iron metabolism. VCNs were proportional to chimeric hemoglobin (human α/mouse β) levels and concurrently HbH was decreased or absent (Fig. 1B). Secondarily transplanted animals showed persistent expression of the therapeutic gene and the beneficial effects observed in primary transplanted mice. Additionally, we tested ALS20αI in erythroid progenitors derived from CD34 cells isolated from patients with both deletional and non-deletional HbH disease, demonstrating reduction in formation of HbH by HPLC (Fig. 1C). In summary, we have developed a new mouse model for α-thal with a lethal and reproducible phenotype. The features of these animals (hypoxia, persistent and high levels of RBC in circulation) are remarkably similar to patients affected by Bart's Hydrops Fetalis Syndrome (BHFS; no α-chains present), rescued by in utero blood transfusion. These data strongly support the use of ALS20αI for the cure of severe forms of α-thal, as the safe level of 2 copies may be sufficient to rescue patients affected by BHFS, while 1 copy may be sufficient to cure patients affected by HbH disease.

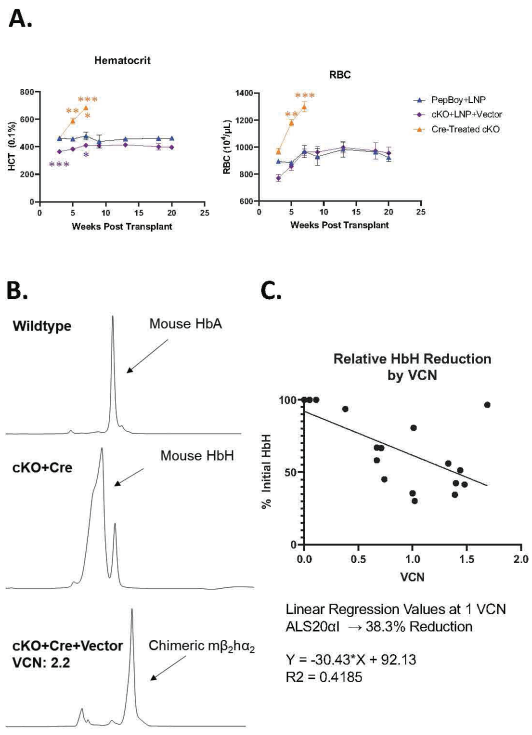

Figure 1: **(A)** Hematocrit and RBC concentration measured in mice transplanted with Cre-treated cKO HSC, Cre-treated cKO HSC with ALS20αI, and Pepboy HSCs. **(B)** Cation Exchange (CATEX) HPLC chromatograms of the listed groups showing the presence of mouse HBA, HBH, and chimeric hemoglobin. **(C)** Relative reduction of HbH peak area by VCN as measured by CATEX HPLC.

70 Liver-Directed Lentiviral Gene Therapy for ARC Syndrome

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Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome is a severe multisystemic autosomal recessive disorder caused mostly by mutations in the *vacuolar protein sorting 33 homolog B* (*VPS33B*) gene. ARC patients display hallmark symptoms including congenital joint contractures, renal tubular acidosis, and neonatal cholestatic jaundice. In the liver, the genetic defect hinders the hepatocytes' ability to secrete several bile components, leading to liver fibrosis, cirrhosis, and terminal-stage liver disease. Whilst liver-directed AAV gene therapy proved efficient in adults with haemophilia, the persistence of AAV expression in the liver of small children may not be sustained long-term due to the episomal nature of the vector. Following breakthrough *ex vivo* treatments of several inherited monogenic disorders, studies on animal models suggest that lentiviral vectors might be safe for *in vivo* use. Thus, we tested the safety and efficacy of an LV.*coVPS33B* lentiviral vector in treating the ARC syndrome's liver phenotype. While *in vitro* tests revealed that lentiviral vector treatment restored *VPS33B* expression and function in a liver cell model, *in vivo* studies in neonate liver-specific *Vps33b*(fl/fl)xAlfp-Cre knock-out mice have uncovered a need for increased hepatocyte transduction for phenotypic improvement. Injections with clodronate-encapsulated liposomes transiently reduce the liver resident Kupffer cell population leading to improved lentiviral vector hepatocyte transduction. Thus, we investigated whether pre-treatment with clodronate could impact the therapeutic outcome in neonatal Vps33b(fl/fl)xAlfp-Cre mice. The mice were fed an 0.5% cholic acid diet at 12 weeks to accelerate phenotype development and sacrificed for analysis a week after. The clodronate-primed mice showed reduced weight loss on the cholic acid diet compared to the mice treated only with the LV.*coVPS33B* vector and improved blood parameters. No safety concerns were identified in 9-months old *Vps33b*(fl/-)xAlfp-Cre mice treated with the LV.*coVPS33B*. These results offer hope for ARC patients and highlight the importance of adequate hepatocyte transduction for lentiviral gene therapy.

71 Improving the Efficiency of *In Vivo* Lentiviral Gene Transfer to Hepatocytes, by Targeting Anti-Viral Pathways, and Application to Familial Hypercholesterolemia

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Lentiviral vectors (LV) are attractive tools for *in vivo* gene transfer to hepatocytes, by virtue of integration in the genome of target cells that allows their maintenance upon cell proliferation during liver growth. We previously showed stable LV-mediated liver gene transfer in neonatal and adult mice, in dogs and non-human primates. However, high quantity of highly purified LV are required to treat large animals and potentially humans. Thus, it would be important to improve the efficiency of LV gene transfer into hepatocytes, in order to reduce the doses required to achieve the therapeutic outcome. This is true for both non-cell-autonomous diseases, like hemophilia - a coagulation disorder due to the lack of coagulation factors - but even more for cell-autonomous diseases, like familial hypercholesterolemia (FH) - due to mutations in low-density lipoprotein receptor, (LDLR) - where most of the liver mass needs to be corrected for the therapeutic outcome. To increase the potency of hepatocyte gene transfer, we focused on anti-viral pathways, targeting them before administering LV. We identified interferon response and proteasome activity as possible targets to enhance transduction. A single dose of either an anti-interferon αβ receptor I (IFNARI) antibody, or bortezomib (a proteasome inhibitor) before LV administration increased transgene output of ~5-fold, both in case of LV encoding for coagulation factor IX or coagulation factor VIII. Interestingly, when tested in combination, we did not observe an additive effect of the two compounds. Bortezomib conferred the highest increase in transgene output, possibly indirectly acting also on IFN pathway by reducing the transduction of plasmacytoid dendritic cells, responsible for IFN production *in vivo*. We then evaluated the efficacy of *in vivo* LV gene transfer in a mouse model of FH, *ldlr*$^{-/-}$. Despite LDLR is reported as the main receptor of vesicular stomatitis virus glycoprotein, VSV.G, used to pseudotype LV, we observed efficient gene transfer in *ldlr*$^{-/-}$ mice by VSV.G-LV, with even higher transgene output compared to wild type mice. Since the lack of LDLR enhanced transduction of VSV.G-LV, we attempted to recapitulate the increase in LV-mediated hepatocyte gene transfer in wild type mice, by reducing LDLR amounts on the hepatocytes' membrane. Fasting is known to downregulate LDLR expression. We thus fasted mice for 24 hours before administering LV, and found transgene output to be up to 7-fold higher than in control mice. We tested the combination of the previously identified enhancers and found fasting + bortezomib to confer the highest advantage, with >11-fold higher transgene output, compared to controls. We then moved to the FH therapeutic experiment in *ldlr*$^{-/-}$ mice. When producing VSV.G-LV encoding LDLR, we obtained very low infectious titers, possibly due to LDLR-VSV.G interaction in producer cells. We solved the issue by avoiding LDLR expression during LV production. With these upgraded LV, we treated juvenile *ldlr*$^{-/-}$ mice and obtained long-term LDL normalization, maintained even following a challenge with a high cholesterol diet, and prevented atherosclerosis of the aorta. Here we report some relatively simple and potentially clinically viable interventions allowing to achieve substantial increase in the potency of *in vivo* LV gene transfer to hepatocytes. Interestingly, reduction of the LDLR in the liver increased rather than decreased LV gene transfer. We exploited this favorable feature to successfully treat FH by liver-directed LV gene therapy in the mouse model.

72 Developement of an *Ex Vivo* Gene Therapy for Infantile GM1 Gangliosidosis

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GM1-gangliosidosis (GM1, OMIM #230500) is a rare, recessive Lysosomal Storage Disorder (LSD) caused mutations in the *GLB1* gene encoding the β-galactosidase (β-gal) enzyme. The enzymatic deficiency induces multiple mechanism of cell damage that finally led to severe, progressive neurodegeneration and premature death of the patients. LSDs require a therapy providing a prompt and robust enzyme delivery to the central nervous system, possibly associated with reduction of local inflammation. *Ex vivo* Gene therapy (GT), i.e. the autologous transplantation of Hematopoietic Stem/Progenitor Cell (HSPCs) genetically-corrected by lentiviral gene transfer, may represent a valuable therapeutic option for GM1, as proven in at least two clinical studies for other LSDs. To this aim, we developed multiple therapeutic lentiviral vectors (LVs) expressing 1) the murine *Glb1* gene, 2) a codon-optimized sequence of the human *GLB1* gene, alone or 3) in combination with the gene encoding the Metallothionein 1G (MT1G), an immunomodulatory molecule potentially contributing to neuroprotection. LV-transduced cells express dose-dependent, supra-physiological levels of therapeutic enzyme, correctly secreted and uptaken by target human hematopoietic cells. In GM1-patient primary fibroblasts, transduction with the therapeutic LVs, both murine and human, determined a stable metabolic correction, proven by the rescue of storage material as early as two weeks post-transduction, with an average of ≥ 3 vector copies/cell. However, the murine β-gal resulted significantly more active than the human enzyme, likely because of a higher stability as described in the literature. We are currently proceeding with the *in vivo* evaluation of our GT strategy in controlling the disease symptoms in a feasibility and efficacy study performed in Glb1$^{-/-}$ mice, animal model of the disease. Preliminary data suggest that the human β-gal-encoding LV restores ~physiological enzymatic activity levels *in vivo* in murine peripheral blood cells, but not the supra-normal activity proven to be essential for enhancing therapeutic benefit in other LSDs. So far, transplantation of HSPCs transduced with this LV resulted in a partial therapeutic efficacy, determining a delay of disease onset and a partial correction of the neuromuscular phenotype of the affected mice. On the other hand, the therapeutic LV expressing the murine β-gal induced supra-physiological enzymatic activity in GM1 mice, with the potential to more robustly benefit the GM1 symptomatology in the animal model. In summary, the LV expressing

the human β-gal is able to achieve supraphysiological enzymatic activity and correct the biochemical phenotype in GM1 patient-derived primary fibroblasts, while the LV expressing the specie-specific isoform of the enzyme seems to be require to correct the GM1 phenotype in the animal model, and validate our GT approach to the disease.

73 Lentiviral Gene Therapy for CARD9 Deficiency

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CARD9 is a critical adaptor protein in the innate immune response to fungal pathogens. CARD9 is phosphorylated downstream of C-type lectin receptors such as Dectin-1 and associates with BCL10 and MALT1 to initiate a signaling cascade resulting in inflammatory cytokine production via NF-KB induction. CARD9 deficiency is an inborn error of immunity characterized by persistent and invasive severe fungal infections of the skin, subcutaneous tissue, mucosal surfaces, and central nervous system. While allogeneic hematopoietic stem cell transplantation can be effective, it is high risk in the context of infection and necessitates a matched donor. The use of a lentiviral vector for *ex vivo* correction of the patients own hematopoietic stem cells(HSC) could be curative while bypassing the need for HLA-matched donors, the use of immune suppressants, and avoiding the risk of graft-versus-host disease. We developed a CARD9 lentiviral vector(LV) and assessed its efficacy in vitro for the treatment of CARD9 deficiency in patient HSCs. CARD9 protein expression was under the control of a constitutive EF-1α promoter. Cryopreserved patient stem cells were thawed and subsequently transduced with 10% diluted vector stock CARD9 LV(2.64e7 IU/mL) at 1e6 cells/mL along with transduction enhancers. Efficacy of the vector was assessed via flow cytometry for CARD9 protein expression, vector copy number(VCN) via droplet digital PCR and colony forming unit(CFU) assay, as well as post-stimulation TNF-α production by ELISA. Following transduction with CARD9 LV, approximately half of CARD9 patient stem cells(PSCs) expressed CARD9 protein as compared to 9% in non-transduced patient and 24% in naïve healthy donor stem cells at fourteen days post-transduction.(Figure 1) CARD9 protein in LV transduced PSCs had increased production of TNF-α in response to Dectin-1 mediated stimulation of the CARD9 pathway as measured via ELISA. Naïve patient monocytes differentiated from HSC showed minimal TNF-α production in response to OXCA stimulation(10.15 pg/mL) while CARD9 LV transduced patient monocytes showed a significant increase in TNF-α production(46.72 pg/mL) even over healthy donor (19.45 pg/mL)($p<0.001$).(Figure 2) Finally, VCN as measured by droplet digital PCR was 1.08 vector copies/cell in the CARD9 LV transduced cells two weeks post-transduction, and 42% of colony forming units from the CFU assay of the transduced cells were positive for vector insertion. Our results demonstrate the production of an effective lentiviral vector for therapeutic replacement of the functional CARD9 gene in hematopoietic stem cells in vitro and show promise for the development of a CARD9 LV gene therapy approach for CARD9-deficient patients. In vivo mouse studies are in progress.

Fig 1. Day 14 Flow

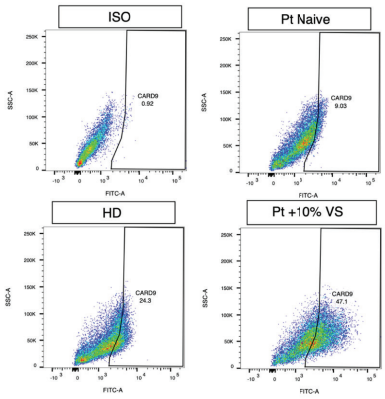
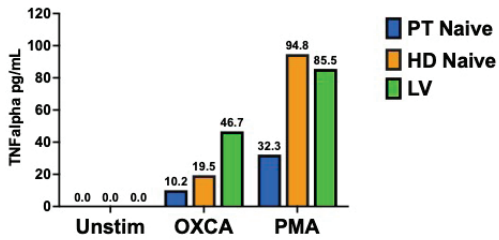


Fig 2. TNFalpha 24h Stimulation



AAV Vectors: Clinical and Non-Human Primate Studies

74 Large-Scale Characterization of the Location and Expansion of AAV Integrations in Macaques and Humans Following *In Vivo* Exposure

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Adeno-associated virus (AAV) vectors are a commonly used delivery vehicle for *in vivo* gene therapies due to the lack of pathogenicity of natural AAV infection and the ability of the AAV genome to persist *in vivo* for a long duration. Researchers have reported observations of the integration of naturally occurring AAV (wild-type AAV [wtAAV]), as well as AAV used in gene therapy applications (recombinant AAV [rAAV]) into host genomic DNA, for over two decades. The possibility of rAAV integration has raised concerns for insertional mutagenesis and clonal expansion in mouse and dog studies; however, no concerns regarding pathogenicity have been raised for wtAAV integration. It remains unknown whether rAAV integration is similar to that of non-site-specific wtAAV integration or whether there is an increased risk of locational genotoxicity or expansion in primates. In this study,

we characterized the genomic location, abundance, and expansion of wtAAV and rAAV integrations in the macaque and human genomes following *in vivo* exposure and assessed the translatability of macaque integration studies to humans. Using a modified unbiased next-generation sequencing technique, we identified the location, number, and distribution of genome-wide AAV integration loci from 168 non-human primates (NHPs) and 85 humans naïve to rAAV exposure, and from 79 NHPs treated with rAAVs via gene therapy studies. Our diverse cohort included samples from rAAV studies with different liver-specific promoters/enhancers (including A1AT and TBG), transgenes (including self, non-self, and human), and capsid serotypes (including AAV8, AAVrh10, AAVhu37, and AAV3B) administered at varying doses ($3x10^{12}$ to $1.2x10^{13}$ genome copies/kg), with timepoints after rAAV treatment ranging from 7 days to 15 years post-gene therapy administration. Our results suggest that rAAV and wtAAV integrations exhibit similar, mostly random, integration distribution patterns within the primate genome, with a higher frequency in genomic regions vulnerable to DNA damage. Although rAAV has a higher abundance of unique integration loci, wtAAV integration loci exhibit higher levels of clonal expansion. The striking qualitative and quantitative similarity between our naïve NHP and human cohorts emphasizes the translatability of AAV integration studies performed in NHP models and supports their use in risk assessment studies over other biologically divergent preclinical animal models. Additionally, our data support the use of samples from the large, easily accessible wtAAV-infected population (NHPs and humans) to expedite the development and validation of rAAV integration detection and monitoring tools for clinical applications. This detailed characterization of AAV integration in primates has important translational implications for the safety of rAAV as a gene therapy vector and highlights the clinical translatability of NHP AAV integration data.

75 The Safety and Efficacy of Pre-Treatment with Imlifidase Prior to Adeno Associated Virus (AAV)-Based Gene Therapy in Non-Human Primates with Pre-Existing Anti-AAVrh74 Antibodies

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Duchenne muscular dystrophy (DMD) is an X-linked, neuromuscular disease caused by mutations in the *DMD* gene that prevent the production of functional dystrophin protein. Delandistrogene moxeparvovec (SRP-9001) is a gene transfer therapy designed to compensate for missing dystrophin in DMD by delivering a transgene that utilizes an adeno-associated virus (AAV) vector—AAVrh74—and encodes SRP-9001 dystrophin, a shortened, engineered dystrophin protein that retains key functional domains of the wild-type protein. Despite generally low levels of pre-existing immunity to AAVrh74 in humans, anti-AAV antibodies do occur and can impact the safety and efficacy of gene therapies and preclude their use in otherwise eligible patients. Here, we assessed the ability of imlifidase, a unique endopeptidase that cleaves IgG, to lower anti-AAVrh74 antibodies as a potential means of overcoming or reducing pre-existing immunity. This study of imlifidase in female cynomolgus monkeys was carried out in two parts with a reporter construct. Part 1 assessed a single (Day 1) or once-daily dose (Days 1-14) of prednisolone via oral gavage (1 mg/kg/day) as a standard immunosuppressive treatment and intravenous (IV) imlifidase (10 mg/kg/dose, Day 1) followed by IV AAVrh74.CMV.eGFP ($1.33x10^{14}$ vg/kg/dose, Day 3) in both anti-AAVrh74 antibody-negative and -positive animals. Part 2 assessed once-daily prednisolone (Days 1-62 or Days 1-96) and single (Day 1) or repeated (Days 1 and 36) imlifidase or saline control and/or AAVrh74.CMV.eGFP (Day 3 or Days 3 and 38). Animals underwent an observation period of at least 59 days. We assessed biodistribution, expression of eGFP, immunological response (anti-AAVrh74 total antibodies using an enzyme-linked immunosorbent assay and T-cell response to AAVrh74 peptides using an enzyme-linked immunosorbent spot), histopathology, and pharmacokinetics/pharmacodynamics of imlifidase. In brief, treatment with imlifidase prior to AAVrh74-eGFP in animals with pre-existing anti-AAVrh74 antibodies (titer range: 1:800-1:1600) led to a decreased anti-AAVrh74 antibody response. The decrease in anti-AAVrh74 antibodies observed in animals that received imlifidase prior to gene therapy resulted in efficient transduction (as measured by droplet digital polymerase chain reaction) and expression (as measured by immunofluorescent quantification) of AAVrh74.CMV.eGFP relative to animals with the same antibody titers that did not receive imlifidase. These results suggest that imlifidase can permit AAV transduction in seropositive animals. No adverse clinical events or mortality occurred subsequent to dosing with gene therapy, and no adverse immunotoxicological or histopathological findings related to imlifidase pre-treatment were found, including in the reproductive organs. In this proof-of-concept study, imlifidase pre-treatment lowered anti-AAVrh74 total antibody titers, allowing for safe administration of AAV-based gene therapy in seropositive animals. These findings may help enable treatment in patients currently excluded due to pre-existing antibodies against AAVrh74. This research was funded by Sarepta Therapeutics.

76 Bilateral Ixo-vec NHP Tolerability and Efficacy Following a Staggered Dosing Interval between Eyes - Gene Therapy nAMD

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Introduction: Ixo-vec is an AAV.7m8 capsid-based intravitreal (IVT) gene therapy designed to produce robust aflibercept levels and is being developed as a gene-based therapy for neovascular age-related macular degeneration (nAMD). Single IVT administration of Ixo-vec results in durable and efficacious levels of aflibercept as demonstrated in the long-term clinical study, OPTIC. The annual incidence of nAMD occurrence in the second eye is between 4-19%. Thus, an interval of months to years may occur between first and second eye treatment. Due to AAV immunogenicity there is a concern of adaptive immunity and neutralizing antibodies developing after the first dose, creating a tolerability and efficacy concern for second eye treatment. The objective of these nonclinical studies was to evaluate the ocular tolerability,

intraocular aflibercept levels, and immune response in non-human primates (NHPs) administered IVT Ixo-vec to contralateral eyes, with a staggered dosing interval between IVT injections. To understand the inflammation profile -onset, duration, and magnitude - no prophylactic regimen was used. Additionally, for comparison, the ocular humoral immune response was examined in a study of unilateral IVT Ixo-vec administration. **Methods:** Two staggered dosing bilateral studies and one unilateral dosing study were conducted in NHPs prescreened for neutralizing antibodies (Nabs). In the bilateral studies, Ixo-vec was administered IVT at 6E11 vector genomes (vg) per eye or 2E12 vg/eye with a 2-month interval between eye injections. As comparator control arms, Ixo-vec 6E11 vg/eye or vehicle was administered to NHPs bilaterally on Day zero. No prophylactic regimen was used. Ocular tolerability was assessed by ophthalmic examinations, OCT and tonometry. Levels of aflibercept in vitreous (VH) and aqueous humors (AH), as well as Nabs in serum and VH samples were assessed. Terminal ocular tissues were evaluated by histopathology. In the unilateral study 2E12, 6E12 or 2E13 vg/right eye were dosed and serum and bilateral humoral response was assessed. **Results:** Ixo-vec was well tolerated. After bilateral dosing, inflammatory findings in both eyes were limited to mostly mild to moderate self-resolving intraocular inflammation (IOI), with no evidence of exacerbated IOI in second treated eyes compared to first or equivalently dosed unilateral injected eyes. Microscopic findings were limited to mononuclear cell infiltrates of minimal severity. Aflibercept levels in second eyes trended lower compared to first eyes yet were within the predicted therapeutic range found across multiple NHP studies testing a wide range of doses including current clinical trial doses 3E10 vg/eye and 1E11 vg/eye (Human equivalent dose 6E10 vg/eye and 2E11 vg/eye). Animals developed Nabs in serum post Ixo-vec administration to first eyes. Nabs were also detected in the first eye following first dose. In the contralateral eye, Nabs were not detected prior to dosing of that eye. This observation was also made in the unilateral study. **Conclusion:** Staggered bilateral administration of Ixo-vec in NHPs did not result in exacerbated IOI in the second-treated eye and is anticipated to provide therapeutic aflibercept levels. Despite detection of Nabs in serum and injected eyes, Nabs were not detected in un-injected eyes suggesting that eyes prior to injection were largely isolated from the systemic humoral response. Clinical translation of the findings around systemic/ocular humoral response will need to be evaluated in the clinical disease setting of nAMD where there is potentially a reduced blood:retinal barrier. Altogether these nonclinical data set the expectation for tolerability and therapeutic aflibercept levels in clinical evaluation.

77 Monitoring Fetal and Infant Somatic Cell Genome Editing in Rhesus Monkeys with Total-Body PET

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Key issues for translational somatic cell genome editing are to understand the extent of editing in the body at a given time, the specificity of editing for the desired genomic target, if edited cells persist, the impact of editing on the immune system, and the potential for adverse events. Addressing these and related questions substantially benefits from a noninvasive approach that can identify edited cells *in vivo*. Positron emission tomography (PET) can provide three-dimensional quantitative images deep inside the body of radiotracers at subnanomolar concentrations. New, transformative total-body PET can image the entire body simultaneously. The goal of these studies was to use total-body PET to identify gene editing *in vivo* in fetal and infant rhesus monkeys. Time-mated dams were identified as pregnant by ultrasound then screened (seronegative for AAV9 and *Sa*Cas9) and selected for the study. Fetuses (N=12) were administered the imaging vector (AAV9/*Sa*Cas9/HSV-sr39TK) *in utero* using an intrahepatic (IH) ultrasound-guided approach in the late first or early second trimester (10e12 vector genomes [vg]/fetus). After administration of ^{18}F-FHBG intravenously to the dams (3 mCi/kg), imaging of fetuses *in utero* was performed in the second and third trimesters and consistently showed editing in the fetal liver, which was sustained across gestation. Findings were confirmed at the tissue level near term with 8-12% editing in the liver, and with no evidence of adverse effects. The AAV9 imaging vector was also administered in a separate study to infants at ~3 months of age (N=16, 10e13 vg/kg; IV or IH under ultrasound guidance). All infants remained healthy and robust during the study period (up to 3 months post-vector administration). Hematology and clinical chemistry panels were all within normal limits and no adverse events were detected. Infants were administered ^{18}F-FHBG IV (0.3 mCi/kg) for PET imaging at two weeks post-administration then monthly during the study period. Similar to fetal studies, extensive evaluation of the liver at tissue collection showed high levels of transduction and an editing outcome of ~7-12% in the liver. These studies have shown for the first time that total-body PET can noninvasively provide insights on gene-edited cells *in vivo* and with high sensitivity and reproducibility.

78 AAV Serotype Tropism and Editing in Young Rhesus Monkeys

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Recombinant AAV vectors are at the forefront of current gene therapy clinical trials. For example, AAV5, AAV6, AAV8, and AAVrh10 have shown promise for liver-directed gene transfer in Hemophilia A and B clinical trials. In musculoskeletal/cardiovascular-directed gene therapies, AAV8, AAV9, and AAVrh74 have attained efficient gene transfer, and AAV9 has proven useful for expression in brain. Despite the abundant and occasionally discordant data describing AAV serotype tropism in animal models and humans, significant uncertainties remain with regard to the selection of an optimal serotype for targeting specific human tissues; thus, there is a need for a side-by-side comparative analysis of multiple AAV serotypes at a comparable dose. A multiplexed (barcode-based) approach was used that enabled comparative and comprehensive evaluation of AAV serotypes following intravenous (IV) administration in rhesus monkeys. The editing cassette included a ubiquitously expressed, mini-chicken beta actin (CBA) promoter driving *Sa*Cas9 with a unique barcode and an adjacent U6 promoter driving a guide RNA (gRNA) This study compared seven AAV serotypes using systemic (IV) administration and young rhesus monkeys (N=40). The AAV serotypes tested (3B, 5, 6, 8, 9, rh10, rh74) delivered *S. aureus* (*Sa)*Cas9 with gene editing measured by assay of indels or integration of the barcoded AAV at the *PCSK9* (proprotein convertase subtilisin/kexin type 9) target site. The barcoding strategy provided a means to administer AAV vectors either individually or in single combined administrations of multiple serotypes (e.g., AAV9+AAVrh74; range 1x10e13-8x10e13 genome copies [gc]/kg). Infants were screened to confirm they were seronegative prior to vector administration, then monitored for 4 weeks post-administration (e.g., hematology, clinical chemistries, immune responses). Initial studies (N=6) tested vector safety and dose using a luciferase reporter (no editing). Overall, findings indicated: (i) all serotypes provided good levels of transduction for a wide range of tissues but limited selectivity was shown for target tissues based on vector tropism; (ii) RNA expression was noted in most tissues but transduction did not correlate with expression; (iii) systemic (IV) delivery resulted in low levels of editing (≤5%); (iv) adaptive immune responses to all AAV serotypes and/or *Sa*Cas9 were observed in all groups; and (v) all animals remained healthy and robust during the study period although transient liver toxicity was observed particularly with the highest dose studied (combined serotypes; total 8x10e13 gc/kg). These studies confirmed that editing was driven by the level of RNA expression, and that transduction is an imperfect predictor of editing. Studies also showed that AAV9 was preferential for many tissues when administered IV (e.g., AAV9>AAVrh10>AAVrh74 ≥AAV8>AAV6>AAV5~AAV3B). As a result of these and related studies where higher levels of editing was observed, we are addressing comparative tests of Cas9 expression cassettes for optimal editing.

79 The Machine Learning-Guided Fit4Function Platform Quantitatively Profiles the Biodistribution of Peptide-Modified AAV Capsids after a Single Round of Screening in Macaque

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Broadening gene therapy applications requires manufacturable vectors that efficiently transduce target cells in humans and preclinical models. Conventional selections of adeno-associated virus (AAV) capsid libraries are inefficient at searching the vast sequence space for the small fraction of vectors with desirable biodistribution profiles maintained across species. We developed a machine learning (ML)-guided Fit4Function platform that evenly samples the manufacturable sequence space of AAV capsids to generate reproducible screening data for training accurate sequence-to-function models.[1] From a single screen in macaque of a Fit4Function library with 100K peptide-modified AAV9 capsid variants, we identified hundreds of capsids with reproducible enhanced biodistribution to the brain, kidney, lung, muscle, heart and liver. With the high-quality data, we built and validated models that can accurately predict biodistribution to different organs (Fig 1A). In addition, we harnessed Fit4Function to assess combinations of functions that can predict a given trait of interest. We found that human cell and mouse models of liver-targeting function, used in combination, could accurately predict cross-species in vivo biodistribution to the macaque liver. Interestingly, human hepatocyte transduction models were more predictive of macaque liver enrichment compared to the mouse liver biodistribution model (Fig 1B). We accurately predicted and validated capsids with enhanced liver-transduction capabilities across species (Fig 1C). This demonstrated that Fit4Function can assess combinations of functional assays that are predictive of a cross-species function of interest, thus informing pipeline design for gene therapy vector development and directing efforts into screens with more predictive power.

[1]Eid, FE et al. "Systematic multi-trait AAV capsid engineering for efficient gene delivery." *bioRxiv* 2022.12.22.521680.

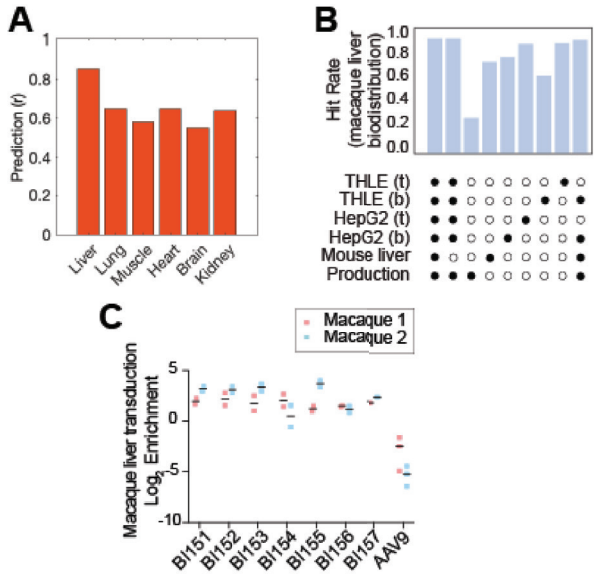

Figure 1. Applying the Fit4Function platform across human cells, mice and macaque to identify multi-trait AAV capsids with cross-species tropisms. (A) The performance of models predicting biodistribution to different macaque organs was measured as the correlation between measured and predicted scores. **(B)** A 100K variant Fit4Function library was injected intravenously into a cynomolgus macaque. Biodistribution was assessed four hours later. The fraction of the indicated variants enriched in the macaque liver (>4-fold more than AAV9) are shown for each combination of predicted traits. Binding or transduction are indicated by 'b' or 't'. **(C)** Fit4Function capsids predicted and validated through individual testing in mouse liver transduction and human cell screens were evaluated in rhesus macaques as part of a pooled library. The macaque liver transduction efficiencies 4 weeks post-injection are shown for the variants, each represented by two amino acid replicates in the library ($n = 2$).

80 AAV-Barcoding for High-Throughput Screening of Vector Transduction Efficiency in Cynomolgus Macaques Compared to C57BL/6 Mice

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Neural degenerative disorders remain a prime target for AAV-based interventions, yet there remains clinical equipoise regarding minutia such as injection methodology and vector capsid characteristics. Moreover, disparities in vector tropism when delivered directly to the brain parenchyma or through the cerebrospinal fluid (CSF) are not well characterized. The clinical translational pipeline for AAV therapy also remains arduous, and many engineered AAV capsids that show enhanced transduction efficiency in mice do not replicate these characteristics in NHP models. Therefore, it is also important to know if there is a correlation of vector transduction dynamics between NPH and mouse models. Here, we set out to characterize vector biodistribution and transgene expression in non-human primate (NHP) and murine models using multiple capsids simultaneously with AAV barcode techniques previously described. Macaques (*M. fascicularis*) and C57/BL6 mice were injected with an AAV barcode mix using either intracisternal magna (ICM) or intraparenchymal (IC6) methods, and multiple analyses were done to reduce unnecessary animal use (*Mol Ther,* 30 115&439, 2022). Here, we demonstrate two such analyses: the characterization of IC6 versus ICM delivery of vector, addressing injection methodology differences, and the disparities between mouse and NHP models -- addressing interspecies differences. After performing rank-order analysis on both biodistribution and expression data organized by anatomical region, the CNS demonstrated a strong correlation ($R>0.70$) in vector rank ordering ($p<0.05$) between species despite visible magnitude differences for individual vectors. A moderate correlation ($R>0.50$) was also noted in non-CNS peripheral organs ($p<0.05$). This was surprising, as there are many cases in the literature where vectors that succeed in mice fail in NHP models, yet at least on a generalized level, there may still be strong correlation between mouse and primate AAV capsid transduction efficiency, at least relative to one another by rankings. Within each animal model, there was strong rank-order correlation ($R>0.70$) of vector capsid types between both IC6 and ICM methods ($p<0.05$), which was expected. Overall, our analysis suggests that rank-orderings of AAV capsids, at least based on the capsids tested in this study, correlate between C57/BL6 murine and *M. fascicularis* primate models. This is significant because it supports the use of mice as a screening tool for effective AAV capsids, since murine models can be powered much more cost-effectively than primates.

Nucleic Acid Therapeutics

81 Transferrin Receptor-Targeted RNA Aptamer Enhanced Blood-Brain Barrier Penetration in Brain Metastases Occurring from Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) is a highly malignant subtype of breast cancer. High invasiveness and heterogeneity, as well as a lack of drug targets, are the main factors leading to poor prognosis. It is estimated that 46% among patients with TNBC will develop brain metastases (BM). BM formation will not only affect the patients' physical function, independence, personality, quality of life, and self-

awareness. Still, it will also significantly increase the mortality rate compared with metastasis to other organs, which can be as high as 80% within one year. The lack of blood-brain barrier (BBB) penetrating ability has hindered the delivery of many therapeutic agents to BM. Herein, transferrin receptor (TfR) aptamer triggered transferrin receptor expressing brain capillary endothelial cells in BBB is subtly developed. TfR aptamer conjugated a small activating RNA (saRNA) targeting the CCAAT enhancer binding protein alpha (CEBPA) gene promotes transcription from target loci, with potential antineoplastic and anti-inflammatory activity. The TfR aptamer conjugated with CEBPa saRNA (TfR-CEBPA) was modified with albumin affinity tag to improve serum stability and validated their efficiency in TNBC-BM humanized mice model. This TfR aptamer with small RNA displays significantly improved brain exposure compared with nano-particle delivery system, as well as the ability to inhibit the tumor growth in mice brain from TNBC. Collectively, results from this study demonstrate that TfR aptamer internalization to delivery across the blood brain barrier into BM, supporting the potential of TfR as a target for drug delivery and antineoplastic activity with CEPBA saRNA in TNBC-BM.

82 The FORCE™ Platform Delivers Oligonucleotides to the Brain in a DM1 Mouse Model and in NHPs

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The FORCE platform was designed to overcome the challenges of oligonucleotide delivery to muscle for the treatment of neuromuscular diseases such as myotonic dystrophy type 1 (DM1). This is a severe neuromuscular disease with prominent manifestations in muscle as well as central nervous system (CNS). DM1 is caused by expanded CUG repeats in the dystrophia myotonica protein kinase (*DMPK*) RNA which sequester muscleblind-like splicing regulators into nuclear aggregates, thereby leading to a spliceopathy that drives clinical progression of the disease. The FORCE platform was optimized to enhance oligonucleotide delivery to muscle and consists of an antigen-binding fragment (Fab) that binds with high affinity to the human transferrin receptor 1 (TfR1) and is conjugated to an oligonucleotide. FORCE conjugates designed to address DM1 contain an antisense oligonucleotide (ASO) that targets nuclear *DMPK* RNA for degradation. Previous work in multiple preclinical models established that FORCE conjugates corrected manifestations of myotonic dystrophy in cardiac and skeletal muscle and demonstrated pharmacological properties that translate from mice to cynomolgus monkeys. It is also important for potential DM1 therapeutics to address CNS manifestations by targeting the mutant *DMPK* RNA in the brain. To determine whether the FORCE platform enables ASO delivery to the brain, hTfR1/DMSXL mice that express human TfR1 (hTfR1) and a mutant human *DMPK* RNA with >1,000 CUG repeats were used as a model of DM1. hTfR1/DMSXL mice were administered intravenously (IV) with either a FORCE conjugate or equimolar doses of unconjugated ASO or a negative control Fab conjugate. The FORCE conjugate, but not unconjugated ASO or the negative control Fab conjugate, delivered ASO to the brain cortex and cerebellum, and the effect was accompanied by suppression of mutant human *DMPK* transcripts and a maximal ~65% reduction of *DMPK* nuclear foci area in brain parenchymal cells. These observations demonstrate that the FORCE platform can deliver an ASO across the blood-brain barrier via TfR1 to drive a robust pharmacological effect in brain parenchymal cells. To establish the translatability of ASO delivery to the brain of higher species, wild-type cynomolgus monkeys were administered an IV infusion of FORCE conjugate or administered with an equimolar dose of unconjugated ASO either by IV or intrathecal (IT) routes. We show that IV dosing of FORCE conjugate delivered up to ~25 to 45-fold higher concentration of ASO compared to IV administration of the unconjugated ASO to cortex and deep brain regions as well as cerebellum. Moreover, ASO detection by *in situ* hybridization in brain sections demonstrated that dosing with the FORCE conjugate leads to widespread delivery of the ASO including the cortex and deep brain regions. In contrast, unconjugated ASO administered IV was not detected, and ASO injected IT remained confined within the most superficial layers of the cerebrum and cerebellar cortex. In conclusion, the FORCE platform delivers ASOs to the brain in preclinical models and has the potential to impact CNS manifestations of DM1.

83 Long-Circulating Lipid Nanoparticles for Agnostic Nucleic Acid Delivery to the Bone Marrow and Beyond

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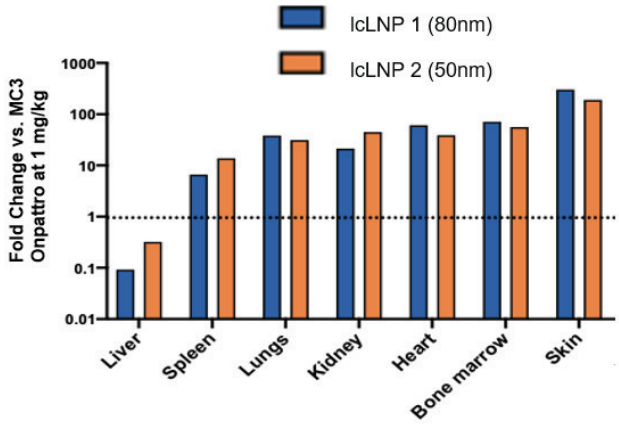
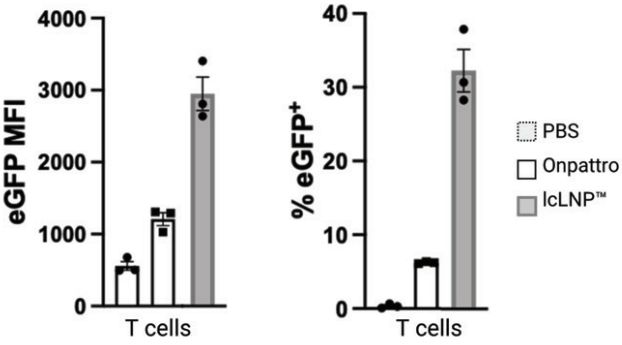
Introduction: Advancements in nucleic acid therapeutics have allowed for broader potential treatment of diseases at the genetic level. However, nucleic acids are prone to degradation by serum endonucleases and clearance through the immune system. Lipid nanoparticles (LNP) have enabled increased potency of these therapeutics by protection through encapsulation and improving their intracellular delivery. However, intravenous administration of current LNP therapeutics is subject to liver accumulation and limited extrahepatic biodistribution due to rapid clearance. Here, we illustrate a novel long-circulating LNP (lcLNP) system capable of extrahepatic delivery to the bone marrow, skin tissue, and solid tumors due to a substantially increased circulation half-life. Methods: LNP formulations consist of ionizable lipid, DSPC, cholesterol, PEG-DMG at differing molar ratios. We use *in vitro* and *in vivo* luminescence and fluorescence quantification assays to assess knockdown or expression in various cell types and isolated tissue samples post-LNP transfection of siRNA, pDNA, and mRNA. We additionally use fluorescence-based lipid dye LNP systems, radiolabelling, and flow cytometry to assess *in vivo* biodistribution and circulation half-life. Results: We have found that our lcLNPs circulate *in vivo* in murine models for greater than 24 hours post-intravenous administration and observe sustained expression up to 5 days. Additionally, our lcLNP systems notably show substantially higher protein expression levels (up to 100X) at later time points in extrahepatic tissues such as the bone marrow, spleen, heart, kidney, and lungs and demonstrate the capability of transfecting skin tissue (Figure 1).



We additionally demonstrate enhanced knockdown of protein expression in abdominal skin tissue and transfection of solid tumours, as well as *in vivo* targeting of T cells within the bone marrow (Figure 2) indicating the disease applicability of our LNP formulations.



Conclusions/Implications: Our lcLNP systems address a key barrier to one of the current issues impeding extrahepatic delivery of genetic payloads and this work demonstrates proof-of-concept experiments that could lead to potential treatment of unaddressed genetic diseases due to current biodistribution barriers.

84 RNA Base Editing for the Treatment of Alpha-1 Antitrypsin Deficiency

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A point mutation in the *SERPINA1* gene (G-to-A mutation, PiZ allele) is the most common cause for Alpha-1 antitrypsin deficiency (AATD). The resulting E342K amino acid change leads to misfolding and aggregation of mutant Z-AAT protein in hepatocytes and a decrease in functional wild-type AAT (M-AAT) in circulation. Thus, this single mutation leads to both toxic gain-of-function and loss-of-function phenotypes, leading to progressive liver injury, lung injury, or both and culminates in end-stage liver and pulmonary disease. Current standard of care, which addresses only the lung manifestation, aims to restore serum M-AAT levels to an anticipated therapeutic threshold (11 μM) by weekly intravenous protein augmentation therapy with plasma-purified AAT. While there are multiple approaches in development to treat AATD, they either address only a subset of patient needs (e.g., silencing approaches to reduce liver aggregates) or do not restore M-AAT (e.g., small-molecule approaches). We aim to decrease Z-AAT and restore M-AAT protein by correcting the PiZ mRNA with AIMers—chemically modified oligonucleotides that direct A-to-I base editing in RNA. Our AIMers incorporate phosphoryl-guanidine (PN) chemistry on a stereopure backbone and recruit endogenous adenosine deaminase acting on RNA (ADAR) enzymes. This approach is expected to preserve physiological regulation of M-AAT while protecting the lungs and decreasing Z-AAT protein aggregation in liver. To facilitate delivery to hepatocytes, we conjugated AIMers to *N*-Acetylgalactosamine (GalNAc). In the NSG-PiZ mouse model that expresses the human PiZ allele, GalNAc-AIMers direct significant PiZ editing in hepatocytes and durably increase serum AAT protein levels. With loading dose followed by bi-weekly dosing, serum AAT levels are maintained at concentrations >11 μM and reach a peak concentration of ~20-30 μM. Secreted AAT protein has the expected wild-type amino acid sequence and inhibits neutrophil elastase. Over time, treated mice show a decrease in liver inflammation and hepatocyte turnover, and reduction in the size of PAS-D positive globules. Lastly, we show that GalNAc-AIMers support dose-dependent PiZ mRNA base editing *in vitro* in primary and iPSC-derived human hepatocytes deficient for M-AAT (MZ heterozygous and ZZ homozygous lines). These findings highlight the potential of GalNAc-AIMers as a therapeutic approach to address both liver and lung manifestations of AATD.

85 Phosphoryl Guanidine-Containing Oligonucleotides Support Exon Skipping in Skeletal Muscle in Mice and Boys with DMD

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Introduction: DMD is the most common genetic muscular dystrophy caused by mutations in the gene encoding dystrophin. Despite numerous FDA approvals, DMD remains a relentless disease, where lack of dystrophin protein results in severe, progressive muscle atrophy and premature death. WVE-N531 is an investigational stereopure antisense oligonucleotide being developed as a potential therapy for patients with DMD amenable to exon 53 skipping. WVE-N531 was designed based on stereopure oligonucleotides that exhibited improved pharmacology compared with phosphorothioate (PS)-modified oligonucleotides in mouse models for DMD. **Methods:** In preclinical experiments, *mdx23* or *mdx/utr*$^{-/-}$ double knockout (dKO) mice were treated with oligonucleotides by intravenous or subcutaneous injection, respectively. Oligonucleotide muscle concentrations (hybridization ELISA), exon skipping (RT-qPCR/TaqMan), and dystrophin (capillary western immunoassay) were quantified as indicated. Three ambulatory boys participated in an intra-patient dose-escalation clinical trial (NCT04906460). The boys received single-escalating doses of 1, 3, 6 and 10 mg/kg; in the multidose portion of the study, the same boys received three doses of 10 mg/kg every other week, and a muscle biopsy occurred 2 weeks after the third and final dose. Exon skipping (RT-PCR), dystrophin protein (western blot), and oligonucleotide in tissue (hybridization ELISA) were quantified as indicated. Oligonucleotide in muscle was visualized (RNAscope, *in situ* hybridization). **Results:** In preclinical studies, stereopure oligonucleotides containing PN chemistry increased muscle exposure, exon skipping and dystrophin restoration in dystrophic muscle compared with stereopure oligonucleotides with PS chemistry alone. The PN-containing molecule prevented premature death and improved median survival from 49 days to at least 280 days in dKO mice. In treated mice, restoration in dystrophin expression was delayed in time compared with exon skipping. The levels of dystrophin achieved in diaphragm and heart and rescue of respiratory function of severely affected dKO mice were encouraging, as expression in these tissues may be needed to improve survival. After the 6-week multidose portion of the clinical study, WVE-N531 reached a mean concentration of 42 µg/g in muscle tissue, with RNAscope providing evidence that WVE-N531 reached the nucleus. Mean exon skipping was 53% (range, 48-62%). Mean dystrophin production was 0.27% (BLQ) of normal. Adverse events were all mild, except for a COVID-19 infection of moderate intensity. There were no serious adverse events, no concerning trends in laboratory data, and no oligonucleotide class-related safety events. **Conclusion:** Preliminary clinical data provide evidence that WVE-N531, which is the first exon-skipping oligonucleotide containing PN chemistry to be tested in the clinic, is leading to substantial exon skipping after three biweekly doses. These data suggest PN chemistry may be impacting pharmacology at the level of human muscle tissue. Although measured dystrophin in patients was low, it is expected that dystrophin production would lag RNA splicing. Extended dosing and follow up are needed to confirm increased production of dystrophin in patients over time.

86 A Novel Small Molecule-Aptamer Pair for Regulating Gene Expression *In Vitro* and *In Vivo*

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Background: Chemical regulation of gene expression by a small molecule via an RNA molecular recognition element (aptamer) enables dynamic and precise tuning of therapeutic gene expression from viral vectors and in genetically engineered cells. Such "riboswitches" offer advantages such as small genetic footprint and lack of immunogenic protein components over the conventional gene switches based on transcription factors. However, the variety of the available small molecules and their cognate aptamers that have been reported to function in mammalian cells and animals are limited. Existing systems (e.g., theophylline, tetracycline, guanine) require >0.1 mM of the trigger molecule to regulate gene expression, limiting their potential biomedical applications. Although RNA aptamers that bind small molecules are not rare, most of them fail to either function in mammalian cells or exhibit a suitable structural change necessary for riboswitch development. **Results:** We set out to generate and screen new aptamers against a panel of pharmaceutically relevant small molecules for mammalian riboswitch applications by SELEX. This effort resulted in a 45-nt RNA aptamer AC17-4 that binds the small molecule ASP2905 with a dissociation constant of 48 nM at 37°C. We observed that, when incorporated into a self-cleaving ribozyme scaffold, the engineered aptazyme is inhibited by aptamer-ligand binding. By inserting the aptazyme in the 3' untranslated region (UTR) of an EGFP transcript, more than 10-fold induction of gene expression was observed in the presence of 5 µM ASP2905 in HEK293 cells transfected with a plasmid vector. The riboswitch was then inserted to an AAV8 vector to regulate human erythropoietin (hEPO) expression in mice. Oral administration of an ASP2905 analog resulted in up to 7.2-fold upregulation of hEPO secretion in serum compared to the control mice. The AC17-4 aptamer was also used to regulate exon skipping following the design strategy developed by another group. The switches showed excellent dynamic ranges (>200-fold induction) with very low basal expression levels in vitro (HEK293 cells). MTT assay showed no detectable toxicity to HEK293 cells up to 10 µM ASP2905. **Conclusions:** The novel aptamer-ligand pair AC17-4/ASP2905 functions at low-micromolar concentrations in mammalian

cell culture systems. In addition to riboswitches, our aptamer-ligand platform may be used to chemically regulate various RNA functions in mammalian cells.

87 tRNA Picovectors Represent a New Therapeutic Approach for the Rescue of Premature Termination Codons

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Background: Mutations which when transcribed convert a sense codon into one of the three stop codons result in a premature termination codon (PTC) in the protein-coding gene sequence. Around 10% of all genetic lesions resulting in human disease are caused by the introduction of a PTC. Disease states caused by PTCs are difficult to address therapeutically because little or no protein target is expressed, both due to direct premature termination of translation and due to the loss of PTC-containing mRNA transcripts through the eukaryotic nonsense mediated decay (NMD) surveillance system. As such, approaches for the therapeutic rescue of PTCs must replace the gene itself or target the aberrant DNA, mRNA, or process of translation. Nonsense suppressor tRNAs or Anticodon Engineered tRNAs (ACE-tRNAs), where the anticodon has been altered to suppress the stop codon causing the PTC, have emerged as a therapeutic avenue to the treatment of disease-causing PTCs. As the translational fidelity of the processes involved in charging the tRNA with its cognate amino acid are retained following the alteration of the anticodon, the PTC is quantitatively reverted to the original amino acid that the protein contained at the site of the PTC. While ACE-tRNAs possess a number of attractive therapeutic properties, they are a fundamentally a gene therapy approach, and as such are subject to the delivery hurdle that all gene therapies face. A major advantage of ACE-tRNAs, however, is the small ~125 bp size of the ACE-tRNA gene including promoter and transcriptional terminator. Encoding ACE-tRNAs as so called 'tRNA picovectors' should impart favorable characteristics to the gene therapy vector including increased cell transfection efficiency, improved intracellular vector trafficking, lower levels of gene silencing, and higher molar equivalents of the cargo sequence per vector delivered. **Methods**: ACE-tRNA picovectors comprising both circular as well as covalently closed linear topologies of 200-400 bp were produced using *in vitro* production methods. *In vivo* recombination based vector production methodologies were also employed for the production of circular ACE-tRNA vectors 850 bp in size. Previously we developed several stable PTC reporter constructs including an mNeonGreen PTC reporter for determining ACE-tRNA delivery efficiency and a NanoLuciferase based PTC reporter for high throughput measurement of PTC suppression efficiency in HBE (human bronchial epithelial) cell lines. Here, we have employed these reporters to determine both the transfection efficiency and PTC rescue efficiency of our ACE-tRNA picovectors. **Results and Conclusions**: We have demonstrated ACE-tRNA function from what are, to the best of our knowledge, the smallest expression vectors used to date. As expected, these ACE-tRNA picovectors display higher transfection efficiency and higher PTC rescue than plasmid DNA in HBE cells. **Acknowledgements**: Funding for this work was provided by a Cystic Fibrosis Foundation Postdoctoral Fellowship to JJP (PORTER20F0) and Cystic Fibrosis Foundation and NIH grants to JDL (LUECK20GO, and NHLBI 1 R01 HL153988-01A1 respectively).

CAR Engineering and Production Advances for Targeting Hematologic and Solid Tumor Malignancies

88 CD5 CAR T-cells Promote Selection of Fratricide-Resistant Circulating T-cells Thus Avoiding Global T-cell Aplasia

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CAR T-cells specific to T-lineage antigens risk ablating normal T-cells resulting in dangerous immunosuppression. Targeting the pan-T cell antigen CD5, broadly expressed in both T-cell lymphoma (TCL) and acute lymphoblastic leukemia (T-ALL) but also by nearly all peripheral T-cells, increases the risk of T-cell aplasia. We have previously reported CD5 CAR T-cells escape self-elimination without additional engineering by undergoing CAR-driven degradation of CD5 protein thus enabling clinical manufacturing of CD5 CAR T-cells for patients with T-cell malignancies. In an ongoing Phase I clinical trial (NCT03081910), CD5 CAR T-cells produce complete tumor elimination in patients with TCL and T-ALL but, unexpectedly, do not eradicate circulating T-cells. The mechanism of this resistance of normal T-cells to CD5-directed cytotoxicity, and whether tumor cells can employ the same strategy to evade elimination, remains unknown. To model clinical findings, we cocultured CD5 CAR T-cells with normal autologous or malignant (T-ALL and TCL) cells. Exposure of normal and malignant T-cells to CD5 CART resulted in a gradual loss of surface CD5. Experiments with engineered dual-tagged CD5 and time-lapse microscopy showed loss of CD5 on target cells was driven by internalization and complete degradation of CD5 mediated by CAR engagement *in trans*. CD5 was re-expressed on target cells within 12-24h of removing CD5 CAR T-cells indicating trans-downmodulation was temporary. Importantly, loss of CD5 temporarily shielded target cells from CD5 CAR T-cell mediated killing but was insufficient to provide long-term protection *in vitro* due to gradual re-expression of CD5 on target cells, thus continuously re-exposing them to cytotoxic CAR T-cells. Despite both normal and malignant T-cells having similar kinetics of CD5 trans-downmodulation upon coculture with CD5 CAR T-cells, healthy peripheral blood T-cells were more resistant to CD5-directed cytotoxicity as a subset of normal T-cells survived a 36-hour coculture with autologous CD5 CAR T-cells while tumor cells were eliminated. Single-cell RNA-sequencing analysis revealed the resistant T-cells were enriched for CD8+ subsets with an effector T-cell program (upregulated cytotoxic effector molecules: granzymes A, B, M, K, perforin, NKG7, KLRB1; downregulated naïve/memory genes: SELL, LEF1, TCF7), consistent with prior reports demonstrating increased resistance of differentiated T-cells to self-directed killing.

To evaluate the contribution of these mechanisms to the resistance of normal circulating T-cells in patients post CD5 CAR T-cell infusions, we analyzed the phenotypic and subset composition of non-transgenic T-cells in peripheral blood. Expansion of CD5 CAR T-cells correlated with a rapid loss of CD5 on circulating T-cells and an enrichment for effector/memory CD8+ populations, mirroring *in vitro* findings. Expectedly, in patients with persisting CD5 CAR T-cells, the CAR-induced trans-downmodulation of CD5 was insufficient for long-term protection, leading to the selection of a non-classical T-cell population lacking CD5 gene expression and enriched for CD8+ alpha-beta and CD4-/CD8- double negative gamma-delta T-cells. These subsets were rapidly replaced with conventional CD5+ T-cells following contraction of CD5 CAR T-cells. Taken together, these results indicate CD5 CAR induces temporary internalization and degradation of CD5 antigen on target cells, but this mechanism provides only short-term protection for normal and malignant T-cells from cytotoxicity. Long-term resistance of normal T-cells in patients having received CD5 CAR T-cell therapy was associated with the emergence of CD5-negative T-cell subsets.

89 CAR Signaling Drives Distinct Immunological Synapse Dynamics That Influence the T Cell Behavior in Killing Cancer

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CAR T cells that signal through CD28 and 41BB are highly effective against lymphoreticular malignancies, despite exhibiting distinct activity patterns in the clinic. We hypothesized that the dynamics of individual CAR T cells at the CAR immunological synapse (CARIS) level, can explain, at least in part, their behavior, *in vivo*. Studying the potency of CAR T cell products targeting HER2 and CD19, we discovered that CD28 polarizes T cells to highly lethal less expansive CD8 effectors. In contrast, 41BB stimulates a Th2 polarizing secretome that supports robust expansion of modestly cytotoxic CD4/CD8 balanced effectors, and persistence of less differentiated central memory T cells. We monitored the single cell behavior of CAR T cells in high throughput, the proteomic composition of the CARIS lipid raft compartment, the mechanical properties and the killing mechanisms of the CARIS, against time. We discovered that CD28 CAR T cells rely on short-lived highly lethal interactions with tumor cells, characterized by brisk shuttling of CARs and their downstream molecules in and out of the CARIS lipid rafts, which leads to rapid MTOC polarization and cytolytic degranulation in a manner that allows for a mastery of serial killing. In contrast, 41BB CAR T cells engage in synapses characterized by slower accumulation of CAR molecules and their associated interactome, and pertinently increased LFA-1 recruitment and retention at mechanically tonic CARIS, in a lengthier, and rather collaborative Fas ligand-mediated killing process. We show here that CAR T cell signaling domains mediate CARIS dynamics that can explain their distinct killing behavior. By understanding and potentially modulating these dynamics, the CAR T cell functionality and toxicity can be rationally optimized.

90 Potent *In Vivo* Transduction by iGPS Particles Generates CAR T Cells with Durable Anti-Tumor Activity

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Adoptive autologous cell therapies have proven to be effective treatments for hematological malignancies. Access to such treatments is severely constrained by the length and complexity of ex vivo manufacturing. Allogeneic approaches are aimed at overcoming scalability and time to treatment but remain limited by inferior clinical responses. A chimeric antigen receptor (CAR) T cell therapy that improves patient access and reduces manufacturing complexity without sacrificing clinical benefit of autologous cell therapies is needed. CAR gene delivery to T cells in vivo is a potential solution that also eliminates the need for conditioning chemotherapy required for cell engraftment. Here we describe an in vivo gene placement system (iGPS™), an advanced lentiviral vector (LVV) particle harboring envelope modifications to improve in vivo gene transfer efficiency and tropism molecules to facilitate T cell-specific gene delivery. When an anti-BCMA CAR transgene was expressed by iGPS particles, low intravenous (i.v.) dose levels caused potent and durable multiple myeloma regression in preclinical models. In vitro culture of normal human peripheral blood mononuclear cells (PBMC) with iGPS particles without T cell-activating antibodies demonstrated concentration-dependent T cell transduction. Even at a low multiplicity of infection of 1, an average of 14% T cell transduction (VCN=1.2) was achieved. Transduction efficiency in vivo was assessed in humanized mouse models after a single i.v. dose of 2e7 TU iGPS particles. An average of 1.9% transduced blood T cells was observed with iGPS particles. Matched doses of "standard" VSVg-pseudotyped LVV particles with and without T cell tropism molecules resulted in 0.27% and 0% transduced T cells, respectively. These data indicate iGPS envelope modifications drive efficient in vivo transduction and preferential outgrowth of T cells. We evaluated anti-tumor activity of anti-BCMA CAR T cells generated in vivo by i.v. treatment with iGPS particles. Complete regression of established multiple myeloma tumors was possible at the lowest dose level tested (1e7 TU). A single dose of 2.5e7 TU iGPS particles resulted in complete tumor control comparable to mice

adoptively transferred with anti-BCMA CAR T cells generated ex vivo using established methods. To evaluate functional persistence of iGPS particle-generated CAR T cells, animals were rechallenged with multiple myeloma tumor cells ~2 weeks after primary tumor clearance. All animals treated with iGPS particles were completely protected against secondary tumor challenge, whereas animals treated with ex vivo-generated CAR T cells showed tumor progression. Off-target transduction was evaluated after infusion of iGPS particles at a therapeutic dose level of 2.5e7 TU in humanized mice. One week following i.v. iGPS administration, a comprehensive set of tissues including heart, brain, ovaries, intestinal tract lung and liver were analyzed for transduction. Across animals, the average T cell transduction was 3.9% and 14% in the blood and spleen, respectively. Strikingly, transduction of other tissues was limited to phagocytes like Kupffer cells, which are expected to nonspecifically take up iGPS particles. No transduction was detected in vital organs or in progenitor cells. iGPS particles can deliver CAR molecules to T cells in vivo with high efficiency and tissue specificity at therapeutic dose levels. Anti-BCMA CAR T cells generated with iGPS particles exhibit anti-tumor potency and functional persistence. These data demonstrate the potential for iGPS particles to be a highly effective, safer, off-the-shelf therapy for patients with multiple myeloma.

91 Engineering Optimal CAR T Cells to Overcome Pancreatic Tumors with Secreted Antagonistic Peptides

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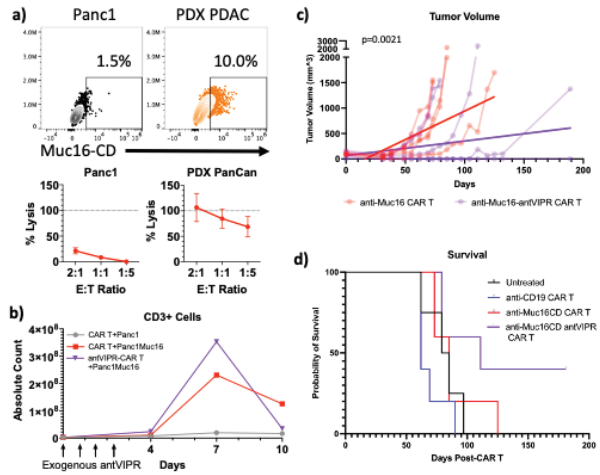
Chimeric antigen receptor (CAR) T cell therapies have shown remarkable clinical efficacy in hematological cancers but still face significant obstacles in the treatment of solid tumors such as pancreatic ductal adenocarcinoma (PDAC). Two major hurdles in developing effective CAR T therapies for PDAC are the identification of ideal tumor-associated antigens (TAA) to target and overcoming a complex tumor microenvironment (TME). We hypothesize that the optimal CAR T cell to treat PDAC recognizes an ideal TAA and protects itself from immune suppression from the TME. Here, we propose the ectodomain of Muc16 (Muc16CD) as a viable TAA expressed in PDAC tumors (Fig 1a) and antagonizing vasoactive intestinal peptide (VIP), an immunosuppressive neuropeptide, to overcome the PDAC TME. First, assayed patient-derived xenograft (PDX) PDAC cell lines were assayed for Muc16CD and VIP expression to establish the clinical relevance of these targets. Interestingly, despite modest Muc16CD expression on PDX lines, Muc16CD-directed 2nd generation CAR T cells had cytotoxic function *in vitro* (Fig 1a) and reduced tumor burden in mice engrafted with orthotopic PDX PDAC tumors. Next, we investigated whether VIP is immunosuppressive for CAR T cell function. VIP limits the proliferative capacity of CAR T cells, which can be reversed by treatment with novel, potent VIPR antagonist peptides. Therefore, anti-Muc16CD CARs were engineered to express antVIPR and provide continuous and localized delivery of antVIPR peptides within the TME. AntVIPR expression by CAR T cells impacts phenotype as these cells have improved cell viability and express less VIP and VIPRs at baseline. Functionally, antVIPR CAR T cells have a proliferative advantage after antigen-stimulation (Fig 1b) and enhanced activation compared to parental CAR T cells. Next, human Panc1 tumors were engrafted into SCID/Beige mice and treated with antVIPR CAR T cells. In this xenograft model of pancreatic cancer, antVIPR CAR T cells reduce tumor burden (Fig 1c) and improve overall survival in mice bearing human VIP-expressing PDAC tumors (Fig 1d). At 118 days, mice were found to have more circulating hCD3+ cells in the antVIPR CAR T-treated mice than the 2nd generation CAR T-treated mice. Collectively, this data demonstrates that the combination of targeting Muc16CD and VIP with a novel CAR T cell can improve anti-tumor efficacy against PDAC. Mouse-specific CAR constructs have been validated to employ in a syngeneic model of pancreatic cancer to determine the mechanisms by which locally secreted VIPR antagonists can modulate the PDAC TME. The long-term goal of translating antVIPR-secreting CAR T cells for the treatment of PDAC.



92 Harnessing CD39 for the Treatment of Colorectal Cancer and Liver Metastases by Engineered T Cells

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Colorectal cancer (CRC) is the 2nd cause of cancer-related death. Despite standard therapies, more than 50% of patients experience relapse, eventually with metastatic disease. Colorectal tumors are densely infiltrated by immune cells that have a role in surveillance and modulation of tumor progression, correlating with improved overall survival. However, exhaustion mechanisms acting within the tumour microenvironment impede their functional capacity against tumor cells. We paired high-dimensional flow cytometry, RNA sequencing, immunohistochemistry and immunofluorescence to describe the T cell functional landscape in tumor and peritumoral tissues from primary colorectal cancers and liver metastases. By CRISPR/Cas9 genome engineering technique, we redirected the specificity of T cells towards a tumor-specific antigen while disrupting inhibitory molecules, to counteract the immune-suppressive tumor microenviroment. Analysis of the healthy, peritumoral and neoplastic tissues of treatment-naïve primary CRCs and of the peritumoral and tumoral tissues of CRC patients undergoing surgery for liver metastasis, revealed extensive transcriptional and spatial remodeling across tumors, being metabolic pathways among the major drivers of this variance. Regarding the immune infiltrate, we found that T cells are mainly localized at the front edge and that tumor-infiltrating T cells co-express multiple inhibitory receptors. Unsupervised analysis of flow cytometry data performed by an advanced pipeline of data handling by dimensionality reduction and clustering algorithms allowed the definition of a peculiar inhibitory receptors signature in TILs enriched both in primary CRCs and liver metastases. Among the highly co-expressed inhibitory receptors, CD39 was found to represent the major driver of exhaustion in both primary and metastatic colorectal tumors. CD39 is a diphosphohydrolase converting ATP into AMP that is emerging as exhaustion marker for tumor-specific T cells, thus highlighting its relevance as molecular target for T cells engineering. By CRISPR/Cas9 genome editing tools, we simultaneously redirected T cell specificity by disrupting the alpha and beta genes of the endogenous T cell receptor with >90% efficiency for both genes, and disrupted CD39 with 100% efficiency, generating triple-knockout engineered lymphocytes. By lentiviral transduction, we redirected the specificity of our engineered T cell product employing a novel T-cell receptor targeting the HER-2 antigen. Triple-edited, HER2-redirected T cells were challenged against HER2+ patient-derived organoids (PDOs) *in vitro* and *in vivo*. Results showed that CD39-disrupted, HER2-redirected T cells have a functional advantage in eliminating CRC PDOs, compared to CD39-competent, HER2-redirected T cells. Thus, CD39 axis is relevant for further exploitation in adoptive T-cell therapy to treat primary and metastatic colorectal cancer.

93 Enhancement of K-Ras Neo-Antigen Targeting CAR-T Cells via Homogenous Knock in of Inducible IL-12

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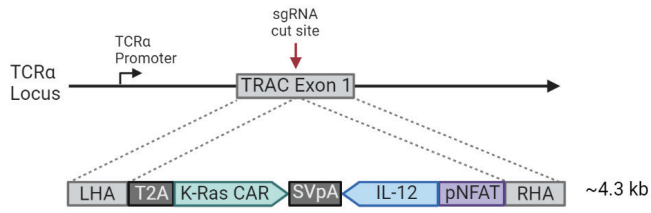
While CAR-T cell cancer immunotherapy has been successful in treating hematological malignancies, there have been significant difficulties in adapting these therapies for the treatment of solid tumors. Challenges include (i) heterogeneity of tumor associated antigens, (ii) insufficient CAR-T cell expansion and cytotoxicity, and (iii) a low therapeutic index, where responding patients experience severe toxicity. We hypothesized that these challenges could simultaneously be addressed with a single CAR-T cell product using our unique genetic engineering approach. To achieve high tumor specificity we developed CAR-T cells targeting K-Ras neo-antigens presented as mutant peptides in MHC molecules on cancer cell surfaces (NeoCARs). We demonstrated *in vivo* efficacy of our NeoCARs against highly conserved G12V mutant peptides in an HLA-A11 dependent context. However, cancer cells typically express 10-100 target neo-antigens each. Low target antigen abundance can result in weakened CAR-T cell activity and failure to achieve a clinical response. In our pre-clinical lung cancer model this resulted in only 43% complete response and survival for the top performing NeoCAR (n=7). We hypothesized that both additional immunostimulatory factors and enhancement of the safety profile are needed expand the therapeutic index for NeoCARs in the treatment of solid tumors. We elected to "armor" our NeoCARs with IL-12 due to its pleiotropic immunostimulatory effects. For an enhanced safety profile and simplified manufacturing of a homogenous product, we developed genetic systems referred to as Uni-Vect. These systems combine antigen induced expression via a nuclear factor of activated T cells (NFAT) response element, with constitutive CAR expression, in a single lentiviral vector. To eliminate aberrant TCR signaling as a source of inducible IL-12 expression, we adapted Uni-Vect for a genome editing approach with homology directed repair (HDR) as a means of template integration. This approach enabled single step generation of TCR disrupted, inducibly armored NeoCARs.



We demonstrate that Uni-Vect enabled homogenous delivery of the constitutive and NFAT inducible modules. Neo-CARs expressing

inducible IL-12 showed an 1-2 log increase in IFN- γ secretion, resulting in upregulation of HLA-A on target cells, and enhanced killing of tumor spheroids *in vitro* ($p<0.00001$). In an *in vivo* model of ovarian cancer we found that inducible IL-12 was necessary for complete clearance of solid tumors, but led to lethal toxicity in all mice receiving TCR positive CAR-T cells (n=5). Only the complete system of CAR, inducible IL-12, and knock in resulted in complete response without lethal toxicity in all mice receiving the treatment (n=5). This coalesced and modular system has broad applicability for the enhancement of next generation of CAR-T cells targeting solid tumors.

94 AMELI-01: A Phase I Trial of UCART123v1.2, an Anti-CD123 Allogeneic CAR-T Cell Product, in Adult Patients with Relapsed or Refractory (R/R) CD123+ Acute Myeloid Leukemia (AML)

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UCART123v1.2 (UCART123) is a genetically modified allogeneic T cell product manufactured from healthy donor cells. Donor derived T cells are transduced using a lentiviral vector to express the anti-CD123 chimeric antigen receptor and are further modified using Cellectis' TALEN® technology to disrupt the T cell receptor alpha constant (TRAC) and CD52 genes to minimize risk of GvHD and allow use of anti-CD52-directed therapy as part of lymphodepletion (LD). AMELI-01 (NCT04106076) is a phase 1 trial evaluating the safety, tolerability, expansion, and persistence of UCART123 given at escalating dose levels after LD with either fludarabine and cyclophosphamide (FC) or FC with alemtuzumab (FCA) in patients (pts) with R/R AML. Key eligibility criteria include pts 18-65 yrs, adequate organ function, ECOG PS ≤ 1, and blasts positive for CD123 by flow cytometry. Pts must have received ≥2 cycles of chemotherapy, ≥ 1 cycle of a high/intermediate dose cytarabine containing regimen, ≥ 2 cycles of an HMA combination regimen, or prior allogeneic HSCT. After LD with FC or FCA, pts received UCART123 at one of the following dose levels in cells/kg: $2.5x10^5$ (DL1); $6.25x10^5$ (DL2); $1.5x10^6$ (DL2i); or $3.03x10^6$ (DL3). The primary endpoint is the safety, tolerability, and MTD/RP2D of UCART123. Additional endpoints include investigator assessed anti-leukemic activity per 2017 ELN criteria, and expansion, trafficking, and persistence of UCART123. As of Oct 10, 2022, 17 pts received UCART123; 8 pts received UCART123 after FC (DL1 [n=2]; DL2 [n=3]; DL2i [n=2]; DL3 [n=1]), and 9 pts received UCART123 after FCA (DL2 [n=8]; DL2i [n=1]). Median baseline BM and PB % blasts were 37% (0-88%) and 19% (0-79%). 5 pts experienced DLTs: 3 pts in FC arm (G4 CRS [1 pt in DL2i and 1 pt in DL3] and G3 ICANS [1 pt in DL2i]); and 2 pts in FCA arm (G5 CRS [1 pt in DL2 and 1 pt in DL2i). Cytokine release syndrome (CRS) occurred in all pts, of which 4 pts experienced ≥ G3 CRS which were classified as DLTs as noted above. Responses were assessed beginning on D28. Evidence of UCART123 activity was observed in 4/16 pts with best overall responses as follows: FC arm (DL2: 1 SD; DL2i: 1 MLFS); FCA arm (DL2: 1 SD and 1 MRD-negative CR). The pt in the DL2 FCA arm with SD achieved greater than 90% BM blast reduction (60% to 5%) at D28. The pt with CR, who failed 5 prior lines of therapy including HSCT and had baseline G4 cytopenias, had CRi at D28 with full count recovery at D56 and remains in an MRD-negative CR beyond the 12 month visit. Adequate LD was not achieved with FC, as host lymphocyte recovery was observed in 7/8 pts prior to D28, and only 3/8 pts had UCART123 expansion. UCART123 expansion was significantly higher with FCA LD (mean UCART123 $AUC_{0\text{-}28days}$ 34.9 ±28.4 days x copies/μg of DNA) vs. FC LD (mean $AUC_{0\text{-}28days}$ 10.2 ± 15.7 days x copies/μg of DNA; p= 0.04). An increase in inflammatory markers, notably serum ferritin, and cytokines with FCA correlated with both UCART123 expansion and CRS. Adding alemtuzumab to FC was associated with improved LD and significantly higher UCART123 cell expansion, which correlated with improved activity, including one pt in the DL2 FCA arm who achieved an ongoing durable MRD-negative CR. Overall, these data support the safety and activity of UCART123 after FCA LD in pts with CD123+ R/R AML. Based on observed UCART123 expansion patterns and cytokine profiles, the study was amended to include a UCART123 2-dose regimen with FCA LD, and enrollment is ongoing into this arm.

Genome Editing Therapies & Safety I

95 Epitope Edited Hematopoietic Stem Cells Allow Immune-Based *In Vivo* Selection of Genome-Engineered Cells

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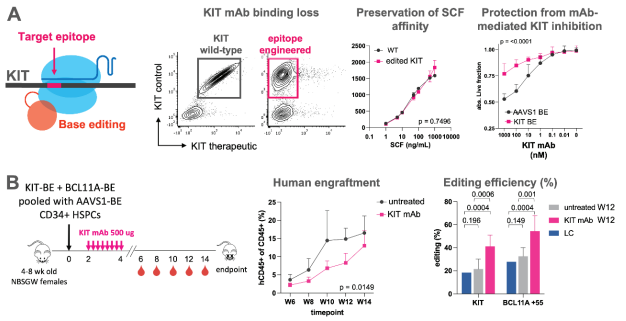
Hematopoietic stem/progenitor cell (HSPC) transplantation (HSCT) offers curative options for patients affected by inheritable diseases for which substitution endogenous hematopoiesis with genetically corrected or healthy donor-derived cells can halt the pathogenic process. Nonetheless, the short- and long-term toxicities of genotoxic conditioning regimens remain a substantial barrier to a wider application of HSCT and gene therapy, in particular for patients with multiple comorbidities. Whereas the use of monoclonal, toxin-conjugated antibodies (mAb) or chimeric antigen receptor (CAR) T cells targeting HSPCs has been proposed as an alternative to chemo/radiotherapy, the mechanism of action and pharmacokinetics of these agents hamper their safe clinical use due to the risk of on-target killing of transplanted HSPCs, short time-window for transplantation,

insufficient clearance of the BM niche and no method to enrich transplanted cells over time. Here, we show that precise editing of the targeted epitope in HSPCs can endow hematopoietic lineages with selective resistance to mAbs or CAR-T, without affecting protein function or regulation. This strategy allows improved myeloablation - which can continue after HSCT - and progressive enrichment of gene-modified HSPCs. By means of epitope mapping libraries, we have identified amino-acid changes in the extracellular domain of KIT (CD117) that abrogate the binding of therapeutic mAbs without affecting surface expression, ligand-mediated activation, downstream signaling and proliferative response (FIG.A). We designed an adenine base editing (BE) strategy to introduce these mutations with high efficiency (up to 80%) in CD34+ HSPCs, with preservation of stem cell phenotype and long-term repopulating and multilineage differentiation capacity in both primary and secondary recipient mice. KITBE CD34+ cells were not affected by the dimerization-blocking effect of the KIT mAb, while expansion of control cells was inhibited in a dose-dependent fashion (FIG.A). To improve HSPC therapies for hemoglobinopathies, such as sickle cell disease, we multiplex base edited the KIT epitope and two BCL11A erythroid enhancer motifs. We obtained high editing efficiencies comparable to single editing on all three target sites and the erythroid progeny of triple-edited cells showed efficient upregulation of HbF. Mice xeno-transplanted with multiplex-edited CD34+ cells showed a 2-fold co-enrichment of both KIT and BCL11A editing in peripheral blood after 2 weeks of Ab administration (experiment in progress), confirming the possibility of selecting gene-modified cells in vivo up to therapeutic thresholds (FIG.B). We envision a new paradigm for non-genotoxic conditioning based on the transplantation of epitope-engineered HSPCs endowed with selective resistance to immunotherapeutic myeloablative agents to achieve safe engraftment and progressive selection of gene-corrected HSPC with substantial advantages over alternatives in terms of safety and tolerability.



96 Development of Prime Editing Strategies for the Treatment of β-hemoglobinopathies

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β-hemoglobinopathies are genetic disorders caused by mutations that reduce adult β-globin production (β-thalassemia) or generate the sickle β^S-globin chain (Sickle Cell Disease, SCD). Genetic correction of autologous hematopoietic stem cells (HSCs) is a therapeutic option for patients lacking a compatible donor. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of genetic mutations termed hereditary persistence of fetal hemoglobin (HPFH), maintaining the fetal hemoglobin (HbF) expression in adult life. γ-globin compensates β-chain deficiency in β-thalassemia and exerts an anti-sickling effect in SCD. HPFH mutations in the promoters of the γ-globin genes (*HBG*) either generate binding sites (BSs) for transcriptional activators (i.e., KLF1, TAL1 or GATA1) or disrupt repressor BSs (i.e., LRF or BCL11A). Prime editing is a novel genome editing tool able to "rewrite" genomic sequences and consists of a prime editor (PE), a prime editing guide RNA (pegRNA) containing the desired edits (DEs) and a nicking guide RNA (ngRNA) that improve editing efficiency (PE3 system). Inhibition of the mismatch repair pathway using a dominant negative form of MLH1 (MLH1dn) favors the incorporation of DEs into the genome (PE5 system). Here, we aim at developing efficient prime editing strategies for β-hemoglobinopathies. In particular, we simultaneously generate multiple HPFH and HPFH-like modifications in the *HBG* promoters to further boost HbF expression. In humans, the co-occurrence of multiple HPFH mutations is associated with higher HbF levels compared to individual mutations. First, we designed multiple pegRNAs and ngRNAs to either (i) insert the KLF1 (-198 T>C) and TAL1 (-175 T>C) BSs, and disrupt the LRF BS in the -200 region or (ii) create the GATA1 (-113 A>G) and the KLF1 (-123; -124 T>C) BSs and disrupt the BCL11A motif in the -115 region of the *HBG* promoters. Plasmid transfection experiments in K562 cells allowed us to select the best performing pegRNAs and ngRNAs that efficiently bind the *HBG* promoters based on the cleavage efficiency induced by the Cas9 nuclease. We then inserted the tevopreQ[1] motif into the pegRNA to increase its stability (enhanced pegRNA, epegRNA) and, as a consequence, the editing efficiency. epegRNAs were tested using the PE3 and PE5 systems into K562 cells. The use of epegRNA and MLH1dn (PE5) improved prime editing efficiency. In the -200 region, we observed insertion either of the KLF1 BS alone or of both KLF1 and TAL1 BSs (up to 13% of promoters harboring both BSs) with a low frequency of InDels. In the -115 region, we observed different profiles using alternative epegRNAs, with ~18% of the promoters carrying only the GATA1 BS and up to 12% harboring both the GATA1 and KLF1 BSs. Interestingly, we also observed impure edits containing not only the BSs but also additional mutations that can be due to imperfect repair of the target region. Finally, we optimized the delivery of the prime editing system in HSCs. We inserted two copies of the *HBB* 3'UTR and a poly-A tail at the 3'-end of the PE mRNA to increase its stability. Furthermore, we tested a variety of parameters including the ratio PE/pegRNA and PE enhancers. Optimization of the different parameters led to increased prime editing efficiency in SCD HSCs upon RNA transfection (~25%). Overall, these results showed that our strategy can simultaneously insert multiple HPFH mutations in the *HBG* promoters. Additional optimizations of the system could improve the purity of the prime editing products and further increase the prime editing efficiency in HSCs with the goal of providing sufficient proof of efficacy for the treatment of β-hemoglobinopathies.

97 CHANGE-seq-BE Enables Sensitive and Unbiased Genome-Wide Profiling of Adenine Base Editors *In Vitro*

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Base editors can correct pathogenic single-nucleotide variants without introducing DNA double-stranded breaks and have tremendous potential to become a new class of safe and effective genomic medicines. However, current methods for understanding the safety and genome-wide activity of base editors have limitations. Some have limited sensitivity as they do not enrich for base editor modified genomic DNA and others are experimentally biased by computational pre-selection of candidate off-target sites. To develop a method for direct, sensitive, unbiased, genome-wide discovery of base editor off-target sites we adapted CHANGE-seq, a scalable biochemical method for defining Cas9 nuclease genome-wide activity, to base editors (**CHANGE-seq-BE**). We used Tn5 tagmentation and exonuclease selection to generate libraries of highly purified genomic DNA circles. CHANGE-seq-BE enables the selective sequencing of adenine base editor modified genomic DNA by enzymatically processing circularized genomic DNA that is nicked and deaminated by base editors into full DNA DSBs for adapter ligation and sequencing. To systematically evaluate the genome-wide off-target activity of ABE8e *in vitro*, we performed CHANGE-seq-BE with sgRNAs targeted to seven therapeutic relevant target sites (*PCSK9*, *HBG1/HBG2* -198, *CD7*, *CIITA*, *PDCD1*, *B2M* and *CBLB*). We found CHANGE-seq-BE read counts were strongly correlated between independent CHANGE-seq-BE technical replicates (R^2>0.9). To validate the sensitivity of CHANGE-seq-BE for identifying sites of *bona fide* cellular off-target base editing, we selected 105 off-target sites from five sgRNA target sites, for analysis by multiplex targeted sequencing in ABE8e base edited cells. Of the 105 CHANGE-seq-BE detected sites we examined, we confirmed 26 (24.7%) by targeted sequencing, with activity ranging from 0.67-88.8%, demonstrating that our method can sensitively detect ABE off-target activity. Next, to determine whether CHANGE-seq-BE could identify base editor-specific off-target activity not detected by nuclease-specific methods, we compared sites identified by CHANGE-seq-BE and by CIRCLE-seq. For a sgRNA targeted to the *HBB gene,* we found that CHANGE-seq-BE identified all known off-target sites as well as an additional ~53% previously unknown *bona fide* off-targets than our initial screen with CIRCLE-seq (Newby et. al, 2021). Furthermore, percentile ranks of validated off-target sites were higher in CHANGE-seq-BE compared to CIRCLE-seq. Taken together, our results demonstrate that direct base-editor specific off-target discovery methods outperform indirect nuclease off-target discovery to comprehensively identify ABE genome-wide off-target activity. In summary, CHANGE-seq-BE enables the rapid characterization of ABE genome-wide activity at a scale not previously achievable by other methods and represents a simple, rapid, sensitive and unbiased method to routinely define genome-wide base editing activity for research and therapeutic applications.

98 Uncovering Upsides and Pitfalls of Base and Prime Editing in Hematopoietic Stem Cells

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Emerging base and prime editing (BE/PE) may provide safer and more precise genetic engineering than nuclease-based approaches bypassing the dependence on DNA double strand breaks (DSBs). However, comprehensive characterization of efficiency, tolerability and genotoxicity of these platforms is lacking and relevant to instruct safe clinical application. Here, we comparatively assessed state-of-the-art cytidine and adenine BE, such as BE4max and ABE8.20-m respectively, and PE versus Cas9 in human hematopoietic stem/progenitor cells (HSPCs) upon mRNA delivery. When targeting a common locus using the same sgRNA, ABE8.20-m outperformed BE4max and Cas9 reaching up to 90% of alleles modification. Cas9-edited HSPCs displayed the lowest engraftment capacity, despite all treatments maintained multilineage reconstitution and no output skewing, as assessed by clonal tracking analyses. Cas9 editing, however, showed moderate shrinkage of clonal complexity in xenotransplanted mice. However, BE4max edited cells tended to decrease over time in the graft pointing to some detrimental response to the treatment in the long-term engrafting HSPC subset. Transcriptional analyses uncovered that BE4max trigger p53 pathway activation, albeit to lower extent as compared to Cas9 treatment, likely due to the conversion of nickase-induced DNA single-SB to DSB. Indeed, sequencing of the target locus revealed that DNA DSBs were less frequent but not abrogated by BE, particularly for the cytidine BE due to suboptimal inhibition of base excision repair. Concordantly, large deletions and translocations were detected upon BE4max treatment despite to lower frequency than upon Cas9 editing. Moreover, ultrahigh coverage whole exome sequencing on long-term xenografts uncovered a genome-wide effect of BE4max on the mutational landscape of hematopoietic clonotypes. Conversely, we did not observe detectable impact of ABE8.20-m in the same analysis. Additionally, both BEs, but not Cas9, upregulated interferon-stimulated genes, suggesting sensing of exogenous long mRNAs. mRNA engineering abolished interferon response without aggravating p53 activation and allowed >80% editing for all platforms in long-term xenografts. In addition, tailored BE4max expression resulted in more efficient BER inhibition leading to reduced proportion of DSBs and improved precision at the target site. We then applied the same mRNA engineering strategy to PE and reached >50% editing in HSPCs. Around 10% of PE alleles carried imprecise editing outcomes, including indels and long-range deletions, despite less frequently than upon Cas9 treatment. Transcriptional analyses showed upregulation of p53- and apoptosis-related genes upon PE. While some p53 targets

were shared by PE and Cas9 treatments, others were specific of PE and likely due to cellular sensing of the reverse transcriptase. Despite these adverse effects reduced the hematopoietic graft size compared to mock electroporated controls, >50% PE efficiency was maintained upon serial transplantation. Overall, our study validated the potential for precise and effective editing of adenine BE among emerging nickase-based platforms, building confidence on its current entry into the clinical arena.

99 Sniper2L, a High-Fidelity Cas9 Variant with High Activity

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Although several high-fidelity SpCas9 variants that have reduced activities at mismatched target sequences have been reported, it has been observed that this increased specificity is associated with reduced on-target activity, limiting the applications of the high-fidelity variants when efficient genome editing is required. Here, we developed an improved version of Sniper-Cas9, Sniper2L, which represents an exception to this trade-off trend as it showed higher specificity with retained high activity. We evaluated Sniper2L activities at a large number of target sequences, and developed DeepSniper, a deep-learning model that can predict the activity of Sniper2L. We also confirmed that Sniper2L can induce highly efficient and specific editing at a large number of target sequences when it is delivered as a ribonucleoprotein complex. Mechanically, the high specificity of Sniper2L originates from its superior ability to avoid unwinding a target DNA containing even a single mismatch. We envision that Sniper2L will be useful when efficient and specific genome editing is required.

100 Genetic Variant Off-Target Editing Assessment by Lentiviral Proxy Assay

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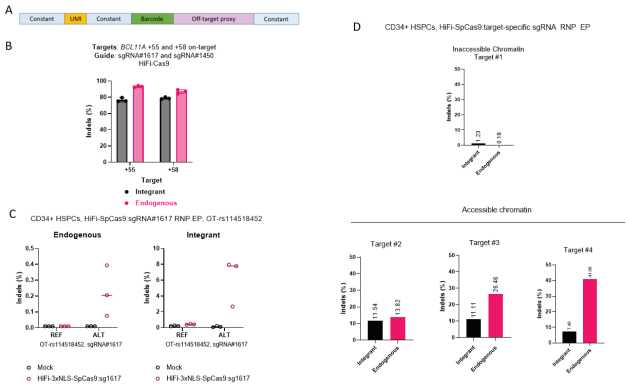
Genetic variation invariably produces allele-specific off-target potential of genome editors. A major challenge for investigating genetic variant-associated off-target editing is that primary cells of relevant genotype are rarely available for experimental validation. Here we describe a proxy assay to transduce HSPCs by a lentiviral (LV) vector carrying a set of user-specified off-target (OT) sequences including alternative-allele specific OTs (Fig A). The LV proxy OT library is integrated to the genome of HSPCs, which may then be subject to a clinically relevant ribonucleoprotein (RNP) electroporation genome editing protocol. After in vitro cell culture for 7-10 days (allowing for decay of episomal lentiviral genomes), genomic DNA is isolated and the proxy off-target sequences are amplified using common primers recognizing flanking sequences and with adapter stubs for amplicon sequencing. First we tested the ability of the assay to identify editing of two on-target BCL11A enhancer protospacers, sg1617 and sg1450, recognizing the +58 and +55 enhancers. We found that the integrant proxies showed similar editing frequency as the endogenous loci across an RNP dose-response range (Fig B). We also found ~1% background indels detected in unedited cells, which could be due to oligo synthesis errors. When we filtered UMIs linked to barcodes and proxy sequences perfectly matching the intended design, background were reduced to less than 0.1%. Next, we tested the ability of the assay to recover off-target edits. We had previously identified an alternative allele specific off-target site for sg1617, associated with the rs114518452 SNP alternative allele C. We had found that editing CD34+ HSPCs heterozygous for the alternative allele with HiFi-Cas9:sg1617 RNP produced 0.2% allele-specific off-target edits. We introduced a lentiviral proxy off-target protospacer in the context of the alternative allele and identified 6% allele-specific edits at the proxy (Fig C). We hypothesized that the heightened sensitivity of editing at the integrant could be due to the endogenous site residing in inaccessible chromatin in CD34+ HSPCs while the integrant proxy would often be in open chromatin due to the nature of lentiviral integration. We tested another inaccessible chromatin candidate OT site with OT matching sgRNA and again found greater sensitivity of the integrant proxy to endogenous target (1.23% indels at integrant proxy as compared to 0.18% indels at the endogenous site). In contrast, we tested 3 sites known to be in accessible chromatin in HSPCs and found at least as great editing at the endogenous sequence as compared to the integrant proxy (13.8%, 26.5%, 41.1% at endogenous vs 11.6%, 11.1% and 7.4% indels at integrant, respectively, Fig D). Finally, we cloned a pool of 80 OT sites, transduced and edited cells, and amplified the pool, showing we could detect all sites in a single amplified pooled proxy library while maintaining sensitivity to detect indels with at least 0.1% frequency. In conclusion, we have developed a sensitive, reliable, and scalable assay that can detect editing potential of pooled synthetic candidate OT sites in clinically relevant primary cells. This allows for validation of genetic variant associated off-target potential in a relevant cellular, editor delivery, and chromatin context while overcoming the limitation of requiring donors of specified genotype.



101 Prime Editing of Human CD34+ Long-Term Hematopoietic Stem Cells Precisely Corrects the Causative Mutation of p47phox Chronic Granulomatous Disease and Restores NADPH Oxidase Activity in Myeloid Progeny

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Chronic granulomatous disease (CGD) is an inherited primary immunodeficiency characterized by low or absent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in phagocytic myeloid cells. Upon sensing pathogens, the NADPH oxidase protein complex produces reactive oxygen species (ROS), that kill the pathogen thus controlling infection. Patients with CGD lack this innate immune response by myeloid cells, which leads to increased susceptibility to, and occurrence of opportunistic infections, chronic inflammatory or autoimmune conditions, impaired quality of life, and decreased life expectancy. The only standard of care disease-modifying approach for CGD is an allogeneic hematopoietic stem cell transplant (allo-HSCT) from a matched donor. However, many patients lack access to matched donors and the risk of complications limits the utility of this approach. p47phox CGD, the most common autosomal recessive form of the disease, is caused by mutations in the *NCF1* gene, which encodes the p47phox protein, a subunit of the NADPH oxidase complex. Approximately 80% of patients with p47phox CGD are homozygous, and most of the remainder heterozygous, for a 2 nucleotide GT deletion (ΔGT) in exon 2 of *NCF1*. This mutation is correctable by Prime Editing (PE). Transplantation of autologous CD34+ cells from p47phox CGD patients in which the ΔGT mutation has been corrected by PE may provide a potential new curative approach. To evaluate this, Prime Editors were developed to precisely correct ΔGT in *NCF1*. Human CD34+ cells were electroporated with PE components and transplanted into NBSGW immunodeficient mice. Sixteen weeks after CD34+ cell transplantation, >84% of long-term hematopoietic stem cells (LT-HSC) that repopulated the bone marrow had at least one *NCF1* allele corrected. No significant differences were observed between 'electroporation only' mock treated control and electroporated Prime Edited human CD34+ cells with respect to long-term CD34+ cell engraftment, human CD45+ blood cell chimerism, hematopoietic multilineage blood cell reconstitution, or LT-HSC cell potency (statistical analyses by ANOVA). Off-target editing events were not detected using a robust, genome-wide suite of assays. In CGD patient CD34+ cells electroporated with PE components, 80% of cells carried at least one corrected allele. These data show that Prime Editing precisely corrects the ΔGT mutation at *NCF1* in p47phox CGD patient CD34+ cells and restores NADPH oxidase activity and myeloid cell function in progeny of these PE corrected cells, thus representing a potential curative approach for p47phox CGD patients.

AAV Engineering for CNS Targeting

102 Structure-Activity Relationships Guided Engineering of AAV Capsids with Optimized Skeletal Muscle, Cardiac Muscle, and CNS Tropism

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Experimental medicines using adeno-associated virus (AAV) to deliver molecular material for muscle and CNS diseases, such as Duchenne muscular dystrophy, genetic cardiomyopathies, and Huntington's disease, hold great promise. For muscle diseases, however, the current medicines use naturally occurring AAV serotypes that require high doses when given intravenously (often >1e14 vg/kg). Reducing these high doses can reduce adverse events, such as liver toxicity, and reduce the high cost of goods sold. For CNS diseases, capsids have limited tropism, particularly for deep brain regions. To overcome these limitations, we have rationally designed a series of new capsids with increased muscle or CNS tropism and reduced liver tropism. Our previous work identified a point mutation (G266A, aka Mut1) that significantly detargeted liver in mice and non-human primates (NHP). When AAV9-Mut1 was combined with a myotropic peptide containing an RGD motif, designated Deco1, the resulting capsid had modestly improved skeletal muscle tropism and reduced liver and heart tropism in NHPs relative to AAV9.To further improve muscle tropism, we tested a combinatorial library of RGD-containing 7-mer peptides inserted at position 588 in AAV9 in NHPs. This screen led to multiple myopeptides with improved muscle tropism compared to Deco1, including myopeptides with >8-fold increased mRNA expression relative to AAV9. In addition, these studies identified myopeptides that specifically increased tropism to either cardiac or skeletal muscle relative to AAV9. Statistical modeling allowed us to determine the structure-activity relationship (SAR) for muscle tropism. Clonal studies in NHPs confirm the results from the library studies showing that the novel capsids had significantly improved skeletal and cardiac muscle tropism (mRNA expression, fraction myocytes expressing cargo) compared to AAV9. Based on the observation that the liver detargeting mutation G266A is in a capsid region that binds to AAVR (VR1), we explored whether additional mutations in the VR1 region would modulate liver tropism while enabling increased muscle tropism. As a result, we identified hundreds of variants using a combinatorial library with liver tropism between AAV9 and AAV9-Mut1 (range of 100-fold mRNA expression) and determined the SAR for liver and muscle tropism. To overcome the limited tropism of CNS capsids, particularly in deep brain regions, we identified a capsid with a median >250-fold increased mRNA expression across different brain regions relative to AAV9 when delivered intravenously using a combinatorial library in NHPs. This capsid lead to widespread CNS expression, including in deep brain regions, compared to AAV9. The structure-activity relationship for CNS tropism was also determined.

In conclusion, we used rational design of combinatorial libraries based on structural modeling and mechanistic hypotheses combined with statistical modeling to deduce SAR relationships for muscle, CNS, and liver tropism. These efforts led to muscle capsids with >8-fold increased mRNA expression in muscle and CNS capsids with >250-fold increased mRNA expression in the brain plus more uniform tissue expression relative to AAV9. Further, these novel capsids have acceptable manufacturing yields and levels of pre-existing immunity to support development to potential medicines.

103 The Use of Transcription-Dependent Directed Evolution (TRADE) to Identify a Novel AAV Capsid That Transduces Motor Neurons with High Efficiency

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Motor neuron diseases (MNDs) are characterized by degeneration of upper and/or lower motor neurons, causing muscle weakness which can lead to paralysis, respiratory failure, and even death. Recombinant adeno-associated virus (AAV) vector-mediated gene therapy is one of the most promising approaches to ameliorate genetic forms of neuromuscular diseases. Although AAV9 has been used clinically for the treatment of MND, the wild-type AAV9 still requires the use of high vector doses (>10E14 vg/kg) to deliver a therapeutic effect. In addition, AAV9 transduces a wide range of cell types effectively, leading to substantial vector spillover to non-target cells in the body. Such a high dosage requirement results in a number of critical issues including off-target toxicities, a limited therapeutic window, and high cost of manufacturing. Thus, the development of an engineered AAV capsid that is efficient in transducing motor neurons is highly desired to effectively treat motor neuron diseases. Here we report that AAV-CGN2, a novel AAV capsid identified by the TRAnscription-dependent Directed Evolution (TRADE) platform, exhibits an enhanced motor neuron tropism following intravenous (i.v.) vector administration in both rodents and non-human primates (NHPs). Cynomolgus macaques and C57BL/6 mice were treated with a single i.v. injection of an AAV-CGN2-CAG-GFP vector. Animals were euthanized 3 weeks after injection and tissues were harvested for downstream histological and biodistribution analyses. To evaluate the efficiency and specificity of motor neuron transduction with the CGN2 variant, we characterized the phenotype of the GFP-positive transduced cells with triple immunolabeling utilizing selective pan-neuronal (NeuN, neuronal nuclei) and motor neuron markers (SMI-32 and choline acetyltransferase, ChAT). Based on previous histological descriptions Betz cells in the NHP primary motor cortex were identified by a set of criteria: their soma size (>600 μm), localization (5b sublayer), proximal dendritic morphology and SMI-32 immunoreactivity. The CGN2 variant demonstrated a significant bias for transducing SMI-32 immunopositive upper motor neurons with minimal glia transduction in both species. In the NHP ventral horn, the ChAT immunostaining revealed that a large number of motor neurons expressed GFP homogeneously all along the ventral horn from the cervical to the lumbar segments. The GFP signal was also present in the axons of the sciatic nerve indicating a transport of the GFP protein along the peripheral axonal pathway. The GFP-positive motor neurons were scored in the cervical and lumbar enlargements that innervate upper and lower limbs, respectively. Motor neuron quantification revealed that >80% of the lower motor neurons expressed GFP in the cervical and lumbar spinal cord segments of the vector-injected NHPs. Notably, no obvious GFP signal was detected in ChAT-negative cells, supporting the motor neuron tropism of the CGN2 variant. Spinal motor neuron transduction was also evident in mice but to a lesser extent compared to NHPs, suggesting possible cross-species differences. Our data demonstrate that TRADE technology can be successfully employed for identification of enhanced AAV variants and that AAV-CGN2 is a powerful vector for the potential treatment of a wide number of motor neuron diseases.

104 AAV-Mediated CNS-Wide Gene Delivery via a De Novo Engineered Capsid-Human Receptor Interaction

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Numerous monogenic central nervous system (CNS) diseases can potentially be treated with AAV-mediated gene therapy. The search for AAV capsids that efficiently cross the blood-brain barrier (BBB) to transduce cells throughout the CNS has predominantly relied on in vivo screens. These can identify rare hits that perform well in the animal model tested. However, it is often challenging to determine whether these capsids will exhibit the same tropism in humans without first elucidating their mechanism of action. Here, we avoided this potential pitfall by directly engineering a suite of capsids that cross the BBB using a known mechanism of action. We screened peptide-modified AAV9 capsid libraries in vitro for their ability to bind to a protein receptor that is highly expressed in human brain vasculature. We identified novel capsids that efficiently transduce CHO cells expressing the human receptor and human brain endothelial cells in vitro. Transduction of human brain endothelial cells by the top performing capsid BI-huBBB1 was inhibited in the presence of a monoclonal antibody against the human receptor. Remarkably, BI-huBBB1 efficiently crossed the BBB in knock-in mice expressing the targeted human receptor, but not in control C57BL/6J mice. In the human receptor knock-in mice, the capsid transduced a large fraction of neurons and glia throughout the brain and spinal cord. These human CNS receptor-targeting AAV capsids are promising vectors for CNS gene therapy applications.

105 Directed Evolution of an AAV9 Library Identifies a Capsid Variant with Enhanced Brain Tropism and Liver De-Targeting in Non-Human Primates and Mice Following Systemic Administration

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A significant limitation of Adeno-Associated Virus (AAV)-mediated gene therapy in the central nervous system (CNS) is the ability of gene transfer vectors to efficiently cross the blood-brain barrier (BBB) upon systemic administration. Recent efforts in directed evolution across the field have yielded AAV variants with marked improvement in translocation across the BBB. However, these variants often only display the increased CNS tropism in the animal species in which the evolution scheme was performed. In an effort to identify capsids with broad species tropism, we applied our RNA-driven TRACER™ capsid evolution platform to evolve Variable Region IV (VR-IV) of AAV9 in two rounds of biopanning in NHPs and mice before synthesizing a library of 1500 top candidates for a third, more focused round of screening in both species. A family of capsids with a distinct consensus motif emerged as the top BBB-penetrant capsids in both mice and NHPs. One member of this capsid family, VCAP-102, was tested individually in African green monkeys (*Chlorocebus sabaeus*) by intravenous dosing of a self-complementary transgene. Across diverse brain regions, VCAP-102 displayed anywhere from 20-fold to 90-fold increased transgene expression relative to wt AAV9. Regions of particularly high tropism compared to AAV9 include the frontal cortex, motor cortex, and cerebellar cortex, putamen, and caudate. In these regions, VCAP-102 transduced anywhere 30 to 50% of cells, targeting both neurons and astrocytes. Notably, VCAP-102 demonstrated reduced expression in both the dorsal root ganglia and liver relative to wt AAV9. Iterative evolution of the VCAP-102 capsid has resulted in a next generation of capsids with upwards of 6- to 7-fold improved BBB-penetrance relative to VCAP-102 in the cynomolgus macaque while maintaining or improving the tropism of VCAP-102 in mice. Taken together, our data presents a novel, CNS-tropic capsid with significantly enhanced BBB-crossing potential, suggesting the likelihood for clinical development.

106 Developing Blood-Brain Barrier-Crossing AAV9 Variants with Reduced Sensitivity to Neutralizing Antibodies

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Pre-existing neutralizing antibodies (NAbs) against adeno-associated virus (AAV) capsids are an obstacle to the success of AAV gene therapies as they limit the range of patients who can receive these treatments. Upon vector administration, NAbs that bind the AAV capsid surface can prevent target tissue transduction and also activate the complement system. We hypothesized that NAbs could be detargeted from the AAV9 capsid via the mutagenesis of carefully chosen surface-exposed residues, without disrupting its blood-brain barrier (BBB)-crossing phenotype. We generated a combinatorial AAV9 capsid library, 'Chameleon', comprising substitutions across 14 distributed, surface-exposed variable residues. We first show that the Chameleon library yields a highly diverse set of capsid variants with a Hamming distance of 1-10 from AAV9, many of which retain their parental production fitness, in vitro and in vivo transduction and thermal stability phenotypes, as well as their compatibility with BBB-penetrating peptides pre-selected in AAV9. The Chameleon library was then subjected to a high-throughput in vitro screen that distinguished capsid variants based on NAb binding. We are currently investigating the production fitness, in vivo transduction and antibody neutralization phenotypes of 12 variants with reduced NAb binding based on our library screen. By combining Chameleon-derived capsid modifications with additional capsid diversification strategies, we seek to develop novel AAV variants with highly reduced NAb binding and desirable in vivo tropisms.

107 Machine-Learning Guided Design of Cell-Specific AAVs in Human Central Nervous System (CNS)

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Introduction:The CNS comprises a multitude of cell types with diverse functionality and specialization. Dysregulation of neuronal or glial populations has been implicated in multiple disorders, including Alzheimer's, Parkinson's, Huntington's disease, etc. AAVs hold tremendous promise as gene delivery vectors to treat such conditions given their reasonable starting efficiency and safety profile. However, such strategies remain challenging due to difficulties in efficient and targeted delivery to specific brain cell populations. Directed evolution can achieve improvements in AAV performance without pre-existing knowledge of virus-cell interactions. Here we apply directed evolution technologies guided by machine-learning (ML) design for developing efficient AAV vectors that target specific CNS cell populations in human

brain tissues, which can serve as basic tools to investigate neuro-glial interactions as well as therapeutic delivery vehicles to treat a range of neurological disorders. Methods/Results:To optimize our library design, we first developed and applied a ML-based framework to design an AAV5-capsid library, focusing on a 7-amino acid insertion region at the 3-fold symmetry axis that was identified as a retargeting site for cell-specific entry. Using the trained predictive model, we designed ~2,238 individual libraries that lie on an optimal curve that trades off predicted enrichment with maximal sequence diversity (**Fig 1A**). To judge the utility of our *in-silico* design approach, we synthesized two of the libraries and showed that our ML-library achieves 5-fold higher packaging fitness than the state-of-the-art library, with approximately 10-fold more successful variants after primary brain infection. From this, we demonstrated that 1) individual ML-predicted AAV sequences exhibited experimental packaging fitness positively correlated to their predictions (**Fig 1B**); 2) synthetic ML-libraries present higher packaging fitness while retaining their high diversity of variants (**Fig 1C**). We then transfected our ML-designed library into packaging cell lines and purified capsid libraries were then administered to human brain slices. Functional selective pressure was imposed by harvesting glial- and neuronal-only cells using MACS 72 hours after infection. Using PCR-based recovery of variants with deep sequencing, we identified converged variants that successfully transduce human glial or neuronal cells specifically (**Fig 1D**). Conclusions:In summary, our approach offers a roadmap for designing an AAV library that broadly improves packaging functionality while retaining high diversity. Our computational method can generate as many libraries as desired by leveraging one predictive model. This approach can be harnessed to optimize libraries for downstream selection for properties of therapeutic relevance, including production, infectivity, specificity, and others. In particular, using our ML-guided library design, we generated novel variants targeting specific CNS cell populations, which will enable future investigation of molecular mechanisms underlying neural disorders and provide potential therapeutics for disease treatment. We also anticipate that such ML-guided design of AAV vectors will have broad utility for the development and selection of novel variants targeting different cells and tissues for applications in the near future.

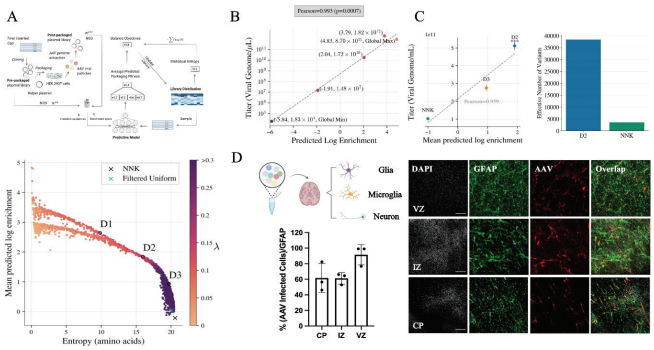

Figure 1: (A) Experimental workflow for generating data for building a ML-based library design using supervised regression model. The predictive model was then systematically inverted to design libraries that trace out an optimal trade-off curve between diversity and packaging fitness. (B) Experimental titers versus predicted log enrichment scores for five synthetic variants. (C) Comparison of ML-designed libraries D2 and D3 to the NNK library. (D) Cell-specific AAVs validation selected from the post-brain infection pool. (Green: Glial Fibrillary acidic protein (GFAP) marker; Red: AAV infected cells; Scale bar = 100 mm; CP: Cortical Plate; IZ: Intermediate Zone; VZ: Ventricular Zone).

108 Functional Gene Delivery to and across Brain Vasculature of Systemic AAVs with Endothelial-Specific Tropism in Rodents and Broad Tropism in Primates

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Delivering genes to and across the brain vasculature efficiently and specifically across species remains a critical challenge for addressing neurological diseases. We have evolved adeno-associated virus (AAV9) capsids into vectors that transduce brain endothelial cells specifically and efficiently following systemic administration in wild-type mice with diverse genetic backgrounds and rats. These AAVs also exhibit superior transduction of the CNS across non-human primates (marmosets and rhesus macaques), and *ex vivo* human brain slices although the endothelial tropism is not conserved across species. The capsid modifications translate from AAV9 to other serotypes such as AAV1 and AAV-DJ, enabling serotype switching for sequential AAV administration in mice. We demonstrate that the endothelial specific mouse capsids can be used to genetically engineer the blood-brain barrier by transforming the mouse brain vasculature into a functional biofactory. Vasculature-secreted Hevin (a synaptogenic protein) rescued synaptic deficits in a mouse model.

AAV Vectors - Product Development Manufacturing: Analytics & Stability Studies

109 *In Vivo* and *In Vitro* Assessment of Residual DNA Impurity-Derived Transcriptions

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Gene therapy products, such as rAAV, are typically produced by DNA plasmid transfected to mammalian cell lines. Host cell DNA and plasmid DNA can be inadvertently packaged inside the rAAV capsids during production. It is important to characterize these residual DNA impurities in gene therapy products due to the theoretical risk of immunogenicity, infectivity, and oncogenicity. Previous studies

showed the level of residual DNA impurity is around 1-2% of the expected rAAV genome. However, it remains unclear whether these DNAs undergo transcription and translation. To address this question, RNA was isolated from transduced cells or mouse liver various days after rAAV transduction, followed by reverse transcription for cDNA synthesis and library preparation. qPCR, ddPCR, and RNA-seq were used to quantify the level of the gene of interest and the impurity genes (Cap8, KanR, Rep2, Ad5 Fiber). Results showed a low but detectable level of Cap8, Rep2, and KanR transcripts. The result from these studies enables targeted risk assessment and better understanding of the potential adverse effects associated with AAV gene therapy. It also points to the importance of improving vector design to reduce the level of DNA impurities.

110 AAV Vector DNA Carrying Two Concatenated miRNA Stem-Loops is Highly Homogenous and Stable in GLP-Grade AAV Product

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MicroRNAs (miRNAs) delivered by recombinant AAV vector (rAAV) are valuable tools in treating gain-of-function diseases by lowering expression of the related genes. However, miRNA stem-loop structure carried in an expression cassette can cause DNA heterogeneity and instability during rAAV production, generating truncated non-functional vectors. AMT-260, our clinical candidate for treating refractory temporal lobe epilepsy, is a single-stranded DNA AAV9 vector carrying two miRNA sequences targeting *GRIK2*, embedded separately in two stem-loop scaffolds. To investigate the genomic integrity of AMT-260, the rAAV was subjected to PacBio single-molecular real-time (SMRT) sequencing. The optimized bioinformatic pipeline increased alignment accuracy, eliminated artefactual sequencing errors and exhibited a highly homogenous rAAV population, with 93% particles containing full-length single-stranded DNA identical to the reference sequence. The results of our meticulous sequencing analysis indicate that high genomic fidelity of rAAV products carrying stem-loop secondary structures can be achieved with a combination of good vector design and using a well-developed manufacturing platform.

111 A Rapid, Serotype Specific Approach to AAV Formulation Development

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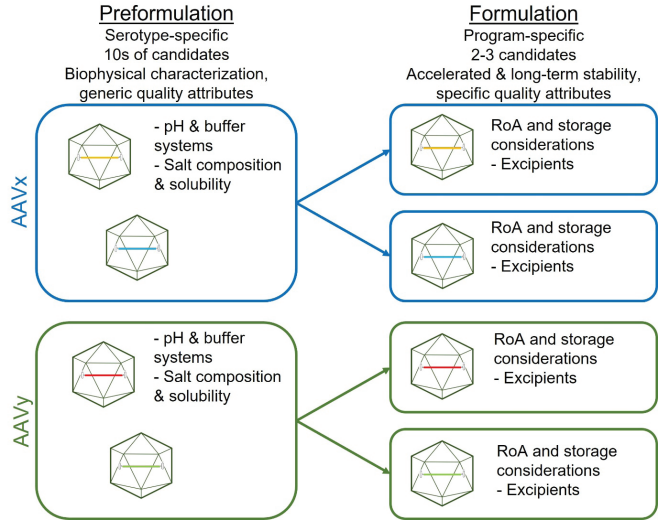
Adeno-associated virus (AAV) is the most widely used vector for gene therapy. Many AAV serotypes, including wild-type and engineered serotypes, are utilized for different tissue tropism and other properties. AAV serotypes are differentiated by protein structure and may each behave differently in aqueous systems. The design space for formulation scientists is vast, and a "generic" formulation is not sufficient to stabilize all AAV serotypes. Compounding challenges include the high cost of goods and variability associated with AAV production as well as the complexity of AAV products which requires evaluation of many quality attributes and large sample-analysis volumes. As such, traditional design of experiment-based formulation approaches are not feasible for AAV, especially at early stages of development. To manage these challenges, a stepwise approach to AAV formulation was developed.



Pre-formulation is performed on each serotype which consists of screening many candidate formulation systems for recovery and stability using generic methods and biophysical characterization techniques requiring small sample volumes. Buffer systems and pH ranges which produce stable formulations as well as ionic strength ranges and salt compositions which yield stable dispersions at high target concentrations are identified. Relative thermal stability parameters of known degradation pathways are compared to identify buffer systems which may yield the most stable formulations over accelerated and long-term stability. Formulation development is then performed for each individual program using information from pre-formulation as well as program-specific design considerations, like routes of administration or patient populations. Stabilizing excipients are evaluated to achieve desired product stability at storage conditions to meet program needs. Larger volumes of fewer candidate formulations are prepared during formulation development. Program-specific quality attributes are evaluated at accelerated stability conditions to select a final formulation. Using the approach described above, we were able to achieve isotonic formulations of AAV1 at up to 2e13 vg/mL with excellent freeze-thaw and accelerated stability at pH 6.9. We also achieved isotonic formulations of AAV2 at up to 1e13 vg/mL at pH 7.5. These accelerated stability results were in agreement with the predictive thermal stability results measured during pre-formulation. Long-term stability is ongoing. Also notable, AAV2 formulated in commonly-used phosphate buffers was highly unstable.

112 Formulate for Success: Early Investment in Drug Product Development Enables Platform Stabilization of Multiple AAV Serotypes, Refrigerated Supply Chain, and Accelerated Dosing Timelines

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AAV preparations have a long-held reputation as difficult to produce, store, distribute, and administer in a clinical setting. As this sector has seen recent growth, a renewed focus on well-developed drug products has become even more critical as regulatory expectations for gene therapies increase. To this end, we have integrated a comprehensive understanding of Drug Product development into our proprietary Process and Manufacturing Platform and applied it to a range of serotypes to demonstrate wide applicability. Our early investment and focus on drug product development allowed us to understand AAV degradation pathways and employ stabilization tactics to design a highly stabilized AAV drug product formulation. Our serotype-independent formulation enables long term storage and shipping at refrigerated temperatures; robustness to freeze-thaw stresses and chemical degradation; prevention of subvisible particulate formation; and aggregation free high-titer products. Our platform formulation has demonstrated long term liquid phase stability in standard titer solutions; high titer stability (>1E14 vg/mL) for at least 1 year at 2-8°C, 3 months at 25°C; and demonstration of liquid stability and aggregation-free solutions at extremely high titers (>1E15 vg/mL). Our proprietary drug product platform offers significant advances for AAV clinical presentations including ease of shipping and storage requirements, reduced vial pooling requirements, and accelerated drug product development timelines for a wide variety of serotypes. Compatibility in our platform formulation has been demonstrated with a wide variety of AAV serotypes in clinical use, including AAV1, AAV2, AAV5, AAV6, AAV8, AAV9, AAVrh10, and AAVrh74. Our streamlined formulation platform evaluation enables rapid identification of serotype compatibility. As an innovation-driven company, OXB Solutions continues to improve its drug product platform to keep our partners ahead of the game.

113 Systematic Comparison of rAAV Vectors Manufactured by Large-Scale Suspension Cultures of Sf9 and HEK293 Cells

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HEK293 and Sf9 cell cultures are broadly used in rAAV production. To study characteristics of rAAV packaged in HEK293 & Sf9 cells, we created self-complementary AAV2.N54-Aflibercept constructs suitable for the production in both systems. Suspension cultures of rBV-Sf9 cells at 2-50 L (Sf9-rAAV) and HEK293 cells (HEK-rAAV) of 2-200 L scales were utilized separately to produce harvest bulks for rAAV purification. The rAAV drug substances (DS) with high purity were systematically characterized including next generation sequencing (NGS), LC-MS/MS analysis, *in vitro* biological activity and *in vivo* efficacy. The integrity of vector genome (vg) was accessed by alkaline agarose gel electrophoresis. Both Sf9-rAAV and HEK-rAAV showed a main vg band plus some minor species in the gel image. SF9-rAAV vg ran slightly lower than vg of HEK-rAAV, likely due to loss of some nucleotides at ITR ends. The culture scale did not affect vg integrity. The rAAV vg contents were assessed by Illumina and PacBio NGS platforms. Interestingly, the resulting vg contents varied between these methods. Specifically, the vg content of Sf9-rAAV was determined at 98.24% and 90.02% by Illumina and PacBio method, respectively, 8% difference between the two methods. The PacBio method was detected at 90.02% and 84.7% for Sf9-rAAV and HEK-rAAV separately, indicating Sf9-rAAV had 5% more vg content than HEK-rAAV. The packed host DNA (HCD) content was measured at 0.03% for Sf9-rAAV and 0.32% for HEK-rAAV by the PacBio method, indicating HEK-rAAV has ~10x more HCD than Sf9-rAAV. The vector DNA chimeric events with HCD were estimated at 2.7% and 10.6% for Sf9-rAAV and HEK-rAAV, respectively. High resolution accurate mass (HR/AM) based proteomic approach was employed to assess the protein level variations. LC-MS/MS protein score plot showed more species at 1500-1800 m/z of the Sf9-rAAV compared to HEK-rAAV, indicating more post translation modifications (PTMs) in Sf9-rAAV. Overlapped and unique PTM sites of rAAVs between the two system are shown in Table 1. **Table 1. Some Commonly Found PTM sites in Sf9 and HEK293 Cell Systems** Furthermore, rAAV infectivity was assessed by the ratio of vg copies per $TCID_{50}$ (I). The vg/I ratios for Sf9-rAAV and HEK-rAAV were at 13-74 and 23-208, respectively. HEK293 and ARPE-19 cells were transduced with Sf9-rAAV and HEK-rAAV vectors in the expression assay. Sf9-rAAV showed 13.6 and 2.1 μg/mL of Aflibercept in HEK293 and ARPE19 cells respectively. HEK-rAAV on the other hand showed 7.3 and 1.2 μg/mL in HEK293 and ARPE19 cells respectively. Final comparison was conducted using the mouse laser choroidal neovascularization (LCNV) model via intravitreal (IVT) dosing with Sf9-rAAV and HEK-rAAV at 4×10^8 and 1.6×10^{10} vg/eye separately. The LCNV model revealed essentially similar anti-inflammation effect. Both rAAV products showed protection at 4×10^8 vg/eye nearly identical to that achieved by 40 μg/eye of commercial product. Complete protection was achieved at 1.6×10^{10} vg/eye. Overall, this work detected notable differences in physiochemical characteristics of rAAV vectors produced by the HEK293 and rBac-Sf9 cell culture systems. However, the *in vitro* and *in vivo* biological functions of the rAAV products from both systems were highly comparable.

Table 1. Summary of the Number of Protein Posttranslational Modification (PTM) sites in rAAV Manufactured in Sf9 and HEK293 Cell Systems (Only some of the commonly found modifications are shown here)

PTM type	Sf9-rAAV	Overlapped sites	HEK-rAAV
Acetylation	4	2	0
GG (ubiquitylation)	4	0	0
Gln>pyro-Glu	0	4	0
Methylation	11	7	3
Oxidation	1	5	0
Phosphorylation	17	3	1
Propionamide	18	14	2

114 Deamidation Analysis for Supporting AAV Gene Therapy Development

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In recent years, recombinant adeno-associated viral (AAV) vectors with nonpathogenic nature and ability to provide long-term gene expression have taken center stage as gene delivery vehicles for gene therapy. Adeno-associated viral capsid proteins (AAV VP) are the major components that determine the tissue specificity and immunogenicity, and play important roles in receptor binding, escape of the virus from the endosome, and transport of the viral DNA to the nucleus. The unique N-terminal region of VP1 is necessary for viral vector infectivity. It was reported that an amino acid mutation from asparagine (N) at 57 to aspartic acid (D) in AAV8 and AAV9 decreased transduction efficiency by more than 50%. Deamidation of asparagine residue is a common post-translational modification of proteins (PTM) and is mostly detected and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based peptide mapping. However, deamidation can be spontaneously introduced during sample preparation prior LC-MS/MS analysis. Traditional sample preparation for AAV VP peptide mapping results in exaggerating the original deamidation levels. It is important to accurately monitor and provide true values of asparagine deamidation for development of AAV gene therapy products. Using AAV serotype 9 (AAV9), we evaluated denaturation temperatures, digestion durations, and digestion temperatures for LC-MS/MS-based assessment of deamidation of capsid proteins. The results demonstrated that the optimal sample preparation method for AAV9 VP peptide mapping minimized asparagine deamidation artifacts significantly. We applied the optimized peptide mapping method and analyzed AAV9 N57 asparagine deamidation on drug substance (DS) stability samples. Increased deamidation in the DS incubated at 25°C for two weeks or one month, or at 2-8°C for 18 months was observed in comparison with the control samples. Although AAV serotype 9 was used for method optimization, due to the high similarity of N57 or N56 containing tryptic peptides from other AAV serotypes, this study provides a general guidance on how to control deamidation artifacts for all AAV serotypes on peptide mapping to support AAV gene therapy development.

115 A Comparative Analysis of Recombinant AAV9 Product Generated from Insect and Mammalian Bioproduction Processes

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Background: In the generation of clinical and commercial-grade gene therapy products, mammalian (HEK-AAV) and insect cell-based (Sf9-AAV) manufacturing continue to be the two predominant recombinant Adeno-Associated Virus (AAV) manufacturing platforms. Each offers its unique advantages and challenges depending on the AAV serotype, transgene design, and material requirement. Insect-based manufacturing is known to provide higher volumetric productivity, whereas transient transfection-based mammalian manufacturing can reduce project timelines associated when switching to a new transgene or a new capsid. In recent years, technical reports have suggested that HEK-AAV is of superior quality and potency than Sf9-AAV. The application of these reports is limited, as these studies utilized Sf9-AAV generated using unoptimized, poorly designed RepCap vectors. In addition, working with the Sf9-AAV platform requires a solid understanding of baculovirus virology, which was not demonstrated in these studies. Neurogene has developed both insect and mammalian cell-based processes as scalable GMP manufacturing platforms. Here, we have generated materials from both processes for a head-to-head analytical comparison. **Methods:** The two optimized, scalable platforms were used to generate AAV9 containing the same transgene of medical importance (GMI) for a direct comparison. Both platforms utilize suspension cell-based production, lysis, clarification, affinity, and Anion-Exchange chromatography (AEX) steps and have been scaled up in our facility from pilot scale to production scale with no impact to volumetric productivity. For this study, AAV9 was generated in single-use bioreactors (SUB). Material generated from these two processes was tested using industry standard analytical methods. **Results:** While the productivity at harvest was higher in Sf9-AAV system (~1e12 vg/mL compared to ~1e11 vg/mL in HEK-AAV9 system), the percent full was similar at harvest for both platforms; and upstream recoveries were comparable. Downstream process also resulted in similar percent full enrichment and removal of impurities in the final product. Finally, the formulated products from both these processes were tested to evaluate quality attributes. Data generated shows that the two processes resulted in products of similar quality based on post-translation modifications, packaging profiles, process related impurities, and *in vitro* potency of products measured by a biological activity assay. **Conclusion:** Using our optimized upstream process, the same downstream process and same GMI, we yield high quality products from both processes with consistently higher productivity from the insect cell-based process.

Genome Editing Therapies & Safety II

116 Pathogenic Exon Deletion Using Prime Editing for Correcting Primary Recessive Dystrophic Epidermolysis Patient Cells

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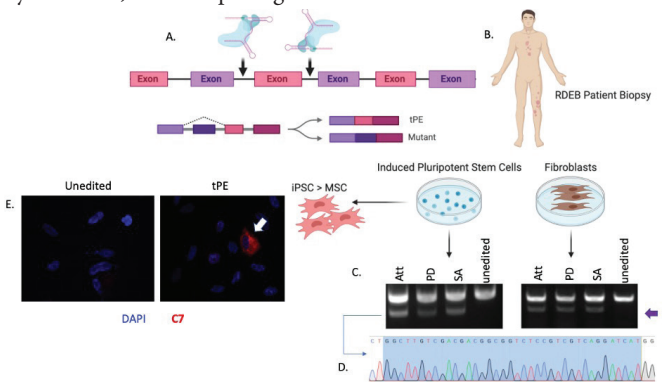
Recessive dystrophic epidermolysis bullosa (RDEB) is a severe mucocutaneous disease caused by loss-of-function mutations to the collagen VIIa (*COL7A1*) gene that encodes the type VII collagen peptide (C7). Previously, we used programmable nucleases and DNA base editors (BE) to repair *COL7A1* mutations through homology-directed repair or precise base editing (BE), respectively. These efforts represent a personalized medicine approach; however, reagents with applicability to multiple patients are desirable. Entire *COL7A1* exons with RDEB causative mutations can be excised to create in frame

deletions leading to restored C7 production. Nucleases that generate double-stranded DNA breaks (DSBs) have been used to accomplish this; however, DSBs can be toxic to the cell and are repaired in a stochastic manner that can limit efficiency. BEs offer gene modification capabilities with low frequency of DSBs; however, they operate through a mechanism not well suited for targeted deletions. Prime editing (PE) is a platform comprised of a Cas9 nickase and an associated fused reverse transcriptase that primes off a user provided prime editor guide RNA (pegRNA). PE enables the ability to install any base substitution and insertions and deletions. Efficiencies of single pegRNA-based PE diminishes as the size of the edit increases. Toward expanding the ability to edit larger sequences, two pegRNAs have been employed flanking a target site that can serve to integrate, replace, or delete large sequences without significant occurrence of DSBs. As such, the use of two pegRNAs represents an ideal strategy for precisely excising entire pathogenic exons such as is observed in *COL7A1* (Fig 1A). We pursued three prime editing strategies with dual pegRNAs in patient-derived induced pluripotent stem cells (iPSC) and fibroblasts (Fig 1B) toward removing a target *COL7A1* exon containing an inactivating premature stop codon mutation: i.) PrimeDel, that generates deletions between paired sites that are nicked by Cas9; ii.) single anchor (SA) that places a fixed deletion at one of the Cas9 nicking sites, and iii.) twinPE (tPE) to replace the target exon with a 38 bp attachment site B (*attB*) from the Bxb1 serine integrase. We observed target exon deletion for each PE approach (purple arrow in Fig 1C). We Sanger sequenced the tPE product and observed *attB* site installation without significant DSBs (Fig 1C; 38 bp *attB* sequence shown shaded in blue, junction is not highlighted). Corrected fibroblasts represent an approach for autologous gene correction and iPSCs can be differentiated in vitro into derivative therapeutic populations for RDEB such as keratinocytes and mesenchymal stromal cells (MSC). TwinPE-editing iPSC-derived MSC demonstrated restoration of C7 production when analyzed by confocal microscopy (arrow/red staining in Fig 1E). The diverse constellation of *COL7A1* mutations makes individual patient specific reagents impractical. Our data shows that tPE is an approach for *COL7A1* correction where a small, well-characterized set of reagents could be employed across numerous RDEB patients to restore C7 expression by DSB-free, in frame pathogenic exon excision.



117 Genome Editing B Cells to Express Custom Heavy Chain Antibodies with Modified Antigen Specificity and Fc Function

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We previously described a genome editing strategy to reprogram the immunoglobulin (Ig) locus of human B cells to express custom Heavy chain antibodies (HCAbs) that recognize specific antigens or other protein ligands. We achieved this by inserting custom antigen recognition domains into the constant region of the IgG1 Heavy chain. This resulted in custom HCAbs that retained essential features of natural antibody function, such as switching from expression of a membrane-anchored (BCR) to the secreted antibody isoform upon plasma cell differentiation. In our initial designs, the constant region of the HCAb was provided by endogenous IgG1 sequences (i.e. Hinge, CH2, and CH3 domains). However, for some applications, it will be desirable to also modify these regions, for example to increase antibody half-life, or enhance effector functions such as antibody-dependent cellular cytotoxicity (ADCC). To achieve these modifications, we expanded our editing strategy to target other sites in the constant region of IgG1, and for other antibody isotypes. In this way, we were able to produce HCAbs with customized Fc domains, such as the GASDALIE mutation in CH2 to enhance ADCC, or the LS mutation in CH3 to increase half-life. By combining these alternate editing strategies and modified Fc domains with different antigen recognition domains that recognize the HIV gp120 protein, we created a series of HCAbs that we could evaluate for optimal anti-HIV activity. Finally, we developed a strategy to accommodate additional domains at the C-terminus of the H chain, while still retaining the ability to produce a BCR isoform. Such a design lends itself to the secretion of engineered molecules such as eCD4-IgG1, whose potent anti-HIV activity is dependent on the presence of a C terminal CCR5 mimetic peptide. We evaluated each HCAb expressed from edited B cells and confirmed that they retained the expected gp120 binding and HIV neutralization activities. This included eCD4-IgG1 secreted from edited B cells, which showed a similar neutralizing activity as recombinant eCD4-IgG1 produced from transfected 293T cells. Ex vivo human tonsil organoids were used to demonstrate that HCAb-edited B cells responded to immunization by the specific HIV gp120 antigen. Finally, the inclusion of the GASDALIE modifications increased killing of HIV-infected target cells by NK cells. In this way, we are able to completely customize all aspects of HCAb design, producing edited human B cells expressing potent anti-HIV antibodies that respond to immunization and that could form the basis of a B cell therapy to suppress HIV replication in vivo.

118 A Targeted Non-Viral CRISPR-Based *In Vivo* Gene Editing Strategy for the Treatment of Sickle Cell Disease

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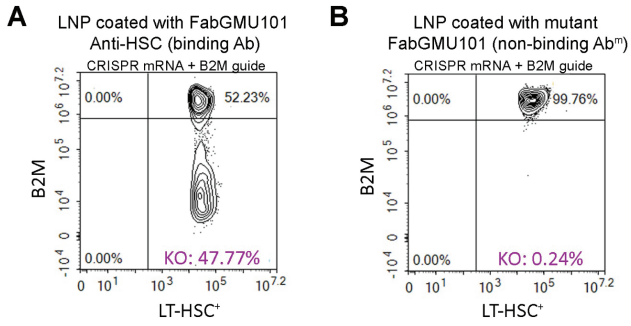
CRISPR/Cas9 has demonstrated tremendous promise to cure sickle cell disease (SCD) by preventing red blood cell sickling and vaso-occlusive events through the genetic modification of BCL11a. Despite the success of clinical studies, there remain limitations. This therapy requires hematopoietic stem cells (HSCs) to be harvested from the patients, genetically modified, and expanded *ex vivo*. While the HSCs are manufactured, patients require treatment with busulfan-based myeloablative conditioning regimens prior to infusion of the genetically engineered HSCs. We have developed a non-viral gene therapy strategy that leverages mRNA, CRISPR editing technology, and targeted lipid nanoparticles (LNPs). This strategy will enable CRISPR nuclease mRNA and an associated guide RNA (gRNA) to be encapsulated by an LNP as cargo. The LNP will then be intravenously injected into a patient, where a targeting moiety will enable specific transfection of HSCs *in vivo*. Once the mRNA cargo is released into the HSCs, the translated CRISPR effector will complex with the gRNA to genetically modify the target resulting in a concomitant increase in the expression of HbF. LNPs were engineered to effectively encapsulate nuclease mRNA and associated gRNA specific for our therapeutic genetic target or the hematopoietic cell-surface markers CD45 or B2M (used as surrogate surface markers to measure editing efficiency). LNPs were then conjugated with an array of antibodies that specifically target cell-surface proteins on HSCs and were screened for binding and transfection on primary human HSCs *ex vivo*. From this screen, one antibody was identified and characterized to optimally transfect HSCs (FabGMU101) (Figure 1). Moreover, we validated the specificity of our strategy by successfully mutating FabGMU101 to block the binding of the surface epitope demonstrating that the editing observed is specifically enabled by FabGMU101 (Figure 1). By incorporating our strategy to enable LNP binding and transfection of nuclease-mRNA, we demonstrate successful disruption of multiple loci in primary human HSCs *in vitro*. This success provides the foundation for a non-viral gene therapy for SCD that will be effective, economically viable, and safe.
Figure legend: HSC targeted-LNP for gene editing. Primary HSC were culture in vitro and treated with LNP encapsulating CRISPR mRNA and beta-2-microglobulin (B2M) guides. LNP are either coated with A) the selected FabGMU101 antibody (binding Ab), recognizing long-term hematopoietic stem cells (LT-HSC) or B) the mutated FabGMU101 (non-binding Abm). The gene editing effects were measured by flow cytometry targeting the cell surface marker B2M.

Figure



119 Precise CRISPR/Cas9 Genome Engineering of Primary Human B Cells Enables a New Class of Cellular Medicines Designed for Sustained Delivery of Therapeutic Biologics

Anja F. Hohmann, Timothy Mullen, Jasmine Edelstein, Sarah Leach, Swati Singh, Alexis Hubaud, Daniel Ferguson, Shivani Bhawsar, Tracy Huang, Connor Malloy, Toshiro Ohsumi, Sean Arlauckas, Richard A. Morgan

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The clinical success of engineered T cell therapies has proven the power of harnessing or re-directing intrinsic cellular functions for therapeutic benefit. Plasma cells (terminally differentiated B cells) offer the possibility to expand the tractable pharmacology of the cell therapy toolkit beyond cytotoxicity. Plasma cells' natural longevity (mean half-life of 17 years) and capacity for high levels of protein secretion (up to 10,000 Ig molecules/cell/sec) make them an attractive source for the sustained delivery of biologics, which require continuous infusion or frequent dosing to achieve a therapeutic benefit. However, until recently B cells were intractable to efficient genome engineering in part due to their resistance to lentiviral vector transduction. Herein, we describe a versatile CRISPR/Cas9-based B cell engineering platform, which allows for the generation of B Cell Medicines (BeCMs) that express and secrete therapeutically relevant proteins at high levels. Transgene knock-in is achieved via HDR-mediated DNA repair, and our platform supports AAV-mediated as well as non-viral delivery of DNA donor templates. Following multiple rounds of optimization of culture and engineering conditions, our platform achieves gene knockouts with greater than 90% efficiency as well as targeted HDR-mediated gene insertions at frequencies as high as 60% without selection. Moreover, the simultaneous editing of multiple genes and knock-in of multiple cargos in a single reaction can be achieved at rates of up to 70% and 25%, respectively. Systematic guide screening identified highly efficient, as well as novel insertion sites including B cell-specific loci that enhanced the amount of cellular protein secretion 5-fold compared to knock-in of the same expression cassette at the CCR5 safe-harbor locus. We also established a clinical guide selection pipeline that allows for the identification of off-target sites in primary human B cells using a combination of *in-silico*, cellular and biochemical off-target discovery techniques. To enable rapid testing of targeted transgene expression constructs, we developed a plasmid-

based screening pipeline in primary human B cells. Using our pipeline, we defined the regulatory elements necessary for optimal transgene expression in plasma cells and can identify the most appropriate coding sequences and signal peptides for the secretion of a given transgene in a matter of weeks. Optimized expression cassettes were then incorporated into AAV vectors and used to engineer primary human B cells with subsequent differentiation into long-lived plasma cells. The robustness of our BeCM platform was demonstrated by the expression of multiple therapeutic proteins such as lysosomal storage disease enzymes, serum proteases, and bispecific T cell engagers. The above-described B cell engineering platform supports the development of autologous as well as allogeneic B cell medicines and allows us to rapidly move from idea to prototype. Novel BeCMs capable of expressing one or more therapeutically relevant transgenes have the potential for broad and meaningful therapeutic utility in rare diseases, cancer, and beyond.

120 Restoration of Full-Length Dystrophin Using AAV-Delivered U7snRNA in Patient Cells with a *DMD* Pseudoexon: A Model for Bespoke Gene Therapies in an Orphaned Mutational Class

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Duchenne muscular dystrophy (DMD) is an X-linked disease caused by a null allele of the *DMD* gene. Thousands of pathogenic *DMD* variants have been described, with deep intronic mutations in the *DMD* gene causing loss of dystrophin expression in 1-7% of all DMD patients. These mutations create cryptic splice sites that mimic canonical splice acceptor and donor sites, resulting in the creation of a pseudoexon, where an intronic fragment is spliced into the coding sequence of the mRNA transcript. Patients with deep intronic mutations possess all of the correct *DMD* coding exons, therefore skipping the pseudoexon should restore a full-length transcript. Objective: To induce expression of full-length dystrophin in cells carrying an intron 18 point mutation, using U7snRNAs targeting either the splice acceptor (SA), splice donor (SD), or exon splice enhancer (ESE) sequences of the resultant frame-shifting pseudoexon. Methods: The AAV-delivered U7snRNA vector repurposes an endogenous U7 small nuclear ribonucleoprotein to induce exon skipping at the pre-mRNA processing step of mRNA maturation. To test for pseudoexon skipping efficacy, human MyoD-transformed cells with a c.2292+1024 G>T mutation were treated with increasing doses of AAV1 containing a single U7snRNA antisense sequence targeting the SA, SD, or ESE. Results: We achieved successful skipping of the pseudoexon, restoring up to 93% of full-length dystrophin mRNA transcript, and 34% of dystrophin protein, as compared to wild-type cells, with the SD and ESE antisense sequences resulting in the most robust skipping response. Conclusions: Our preliminary data confirms the pseudoexon class of *DMD* mutations is amenable to U7snRNA treatment, and we plan to further validate this in other DMD patient cell lines. This approach addresses an orphaned class of *DMD* mutations, and demonstrates the potential of bespoke gene therapies to restore full-length dystrophin in individual patients.

121 Large-Scale GUIDE-seq-2 Profiling Reveals Effects of Human Genetic Variation on Cellular Off-Target Genome Editing Activity

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CRISPR-Cas genome editors hold enormous potential for the treatment of a wide spectrum of genetic diseases and some therapeutic approaches are in ongoing Phase III clinical trials. As gene therapy products that incorporate human genome editing become approved, thousands of patients may to be treated with these new future therapies. An important unanswered question is how individual genetic variation may impact the safety of these treatments. We and others have described examples of point mutations that affect genome editing activity. Here we describe the first, to our knowledge, large-scale experimental studies of the effects of genetic variation within four human populations (ASW, African Ancestry in Southwest USA; CEU, Utah residents with Northern and Western European ancestry; HCB, Han Chinese in Beijing; and MXL, Mexican Ancestry in Los Angeles) on the genome-wide off-target activity of editors. We reasoned that a high-throughput version of GUIDE-seq, a cell-based method for defining nuclease genome-wide activity, could enable the rapid measurement of change in genome editing activity due to genetic variation within on- and off-target sites. We designed and optimized GUIDE-seq-2, a sensitive, streamlined, automated and high-throughput Tn5-tagmentation enabled version of GUIDE-seq which is comparable to the original method and highly reproducible. To determine the impact of single genetic variation on Cas9 off-target activity, using GUIDE-seq-2 we performed a large-scale evaluation of genome-wide off-target activity for six sgRNAs in lymphoblastoid cell lines (LCLs) from ninety-four individuals across four populations characterized by the 1000 genomes project. We measured CRISPR-Cas9 off-target activity across the six target sites and 94 LCL donors using GUIDE-seq-2 aided by an automated liquid handling system. We detected a total of 963 unique off-target sites across the six target sites and 94 LCL donors that we evaluated. Interestingly, we found that 18.7% (180/963) of the off-targets detected, a relatively high proportion, harbored SNPs within at least one individual's genome. We then modeled the effects of genetic variation on GUIDE-seq-2 read counts (a direct measurement of cellular Cas9 activity) and found 15% (27/180) of genetic variants within off-target sites had significant effects on Cas9 activity ($P<0.05$). Variants with strong effects on off-target activity were found at expected protospacer adjacent motif (PAM)-proximal and also less likely PAM-distal regions of the off-target sites. In sum, we found approximately 2.8% (27/963) of all detected off-target sites were significantly affected by non-reference genetic variants, with an average of 32.4% (1.1%-93%) of individuals affected per off-target site. Taken together, our results show that genetic variation can strongly

impact cellular Cas9 genome-wide off-target activity and highlights the importance of considering how this may affect the safety and efficacy of broadly applicable genome editing therapies.

122 Modeling Gene Editing Outcomes in Microphysiological Human Tissue System Models of Duchenne Muscular Dystrophy

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Gene editing has shown potential to treat human diseases, however there remains an unmet need for physiologically relevant preclinical models that can accurately predict the safety and efficacy of gene editors in humans. To address this, we have engineered human skeletal muscle microphysiological tissue systems (myobundles) that model the dystrophic phenotype characteristic of Duchenne muscular dystrophy (DMD). In this study, we report successful editing of the DMD gene using two different myobundle models and three different gene editing strategies delivered via AAV. We show that gene correction leads to significantly improved muscle health and function, demonstrating the potential of myobundles as a preclinical model. We first determined whether myobundles could accurately model the effects of gene editors delivered via AAV. We completed an AAV serotype screen where hiPSC-derived *DMD*Δ48-50 myoblasts were transduced at the time of 3D tissue formation with a panel of AAV serotypes encoding GFP. Based on GFP+ cross-sectional area (CSA), AAV6 had the highest transduction efficiency at 40%, followed by AAV2 at 20%, and AAV8 and 9 had little to no transduction. We then evaluated the editing efficiency of deleting exon 51 by a CRISPR-based editing strategy delivered via two AAV vectors (g51). We observed productive edits in 15% of alleles, which restored the reading frame in 40% of dystrophin transcripts and rescued 12% of wild-type dystrophin protein levels. To further demonstrate the utility of this model, we created an additional myobundle line lacking *DMD* exon 44 and evaluated two exon skipping strategies: an adenine base editor and SpCas9 targeted to the exon 45 splice acceptor. Treatment with the adenine base editor and SpCas9 led to exon 45 skipping in 30% and 6% of transcripts and restoration of 17% and 5% of wild-type dystrophin protein levels, respectively. We also compared the function of myobundles treated with g51 vs. a non-targeting gRNA (gCtrl). Interestingly, we found that g51-treated myobundles had a small, but significant decrease in specific force (contractile force/CSA) compared to gCtrl-treated myobundles. However, g51-treated myobundles showed significantly less reduction of force generation compared to gCtrl after injury, suggesting partial amelioration of the dystrophic phenotype. Further histological analysis will characterize the effect of editing on myobundle health and function. Finally, we treated an immortalized patient myoblast line lacking *DMD* exons 48-50 with g51 and isolated edited cells to create a monoclonal line of uniformly corrected cells (*DMD*Δ48-51), which were then used to make myobundles. Histological analysis showed that *DMD*Δ48-51 had significantly increased myotube diameter and improved sarcomere structure, suggesting improved muscle structure and health, compared to uncorrected controls. *DMD*Δ48-51 myobundles generated a significantly greater specific force and were protected from injury compared to unedited myobundles. Together, these results suggest that restoration of dystrophin reverses the functional deficits seen in dystrophic human myobundles. Collectively, this foundational work shows that 3D microphysiological muscle tissue models can be used to perform safety, efficacy, and functional assays in a model that accurately captures human muscle physiology, directly addressing a need in the gene editing field that has been unmet by 2D cell culture and animal models. Additionally, improvements to the model, including the incorporation of immune cells and vasculature, may continue to increase its ability to accurately predict the safety and efficacy of gene therapies in patients.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases I

123 Oligodendrocyte Toxicity by Guanidino Compounds, Not Arginine, Fosters Dysmyelination in Arginase Deficiency and is Prevented by AAV-Based Gene Therapy

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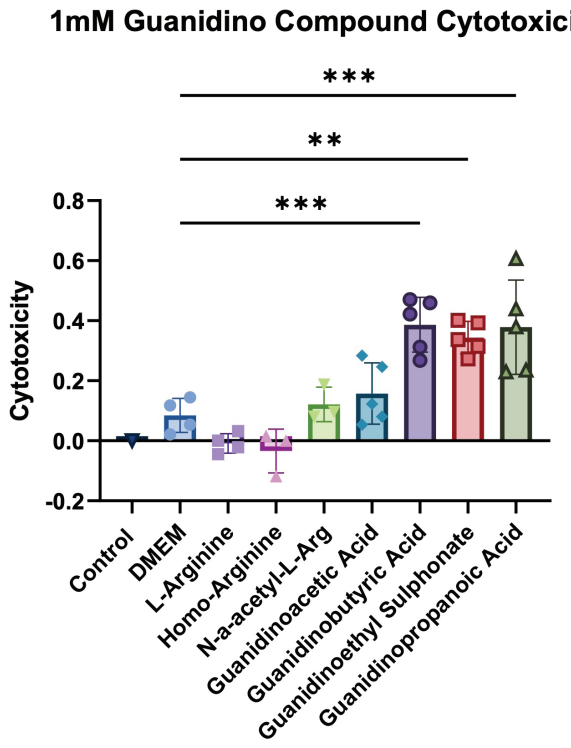
Introduction: Arginase deficiency is a distal urea cycle disorder (UCD) that has unique, characteristic symptoms that differ from other UCDs. These include spastic diplegia/tetraplegia, intellectual disability, & progressive irreversible neurological decline. Recent studies have demonstrated that central nervous system (CNS) dysmyelination is a prominent feature of the disorder & may in part be the cause of the unique neuromotor phenotype. While also classified now as a leukodystrophy, the underlying cause of oligodendrocyte dysfunction in arginase deficiency is unclear. We hypothesized that either hyperargininemia itself or substances related to arginine metabolism (i.e., guanidino compounds, which are generally related to transamidination by the promiscuous enzyme L-arginine-glycine amidinotransferase) may be the cause of the observed dysmyelination phenotype. In this study, we investigated the potential mechanisms of myelination dysregulation in arginase deficiency & have shown that dysmyelination can be prevented by AAV-based treatment. **Methods/Results**: Microarray analysis suggested alteration of oligodendrocyte function by postnatal day (P) 15 & histological markers in the brain supported this finding. Utilizing electron microscopy & murine model of arginase deficiency, we found a lack of myelination during beginnings of CNS model myelination in untreated Arginase 1 knockout mice. Myelinated axon density in subcortical white matter was significantly lower in untreated Arginase 1 knockout mice than Wild Type at P15. Myelinated axon density in the pyramidal tract was also significant lower in untreated Arginase 1 knockout mice when analyzed at P6, P10 & P15. To restore Arginase expression, we generated a serotype rh10 AAV expressing human codon-optimized Arginase 1 under a TBG promoter; 1e14 GC/kg was administered intravenously on P2. Analysis demonstrated that AAV treatment successfully restored myelinated axon density to a level comparable to wild type in both brain regions by 4-months. We isolated oligodendrocyte progenitor

cells (OPC) through antibody-based cell selection & exposed them to guanidino compounds. Guanidino compound treatment at concentrations of 1mM & 3mM resulted in significant cell death. In contrast, exposing OPCs to arginine at both concentrations did not induce changes in cell viability (Figure 1). We further hypothesized that guanidino compounds enter OPCs through the creatine transporter SLC6A8 due to similarity in structure between guanidino compounds & creatine. SLC6A8 overexpression in HEK293T cells demonstrated competition between creatine & several guanidino compounds as assessed by a creatine uptake assay. Supplementation of media with creatine resulted in greater survival of OPCs, suggesting a potential mechanism how elevated guanidino compounds could cause OPCs' death. **Conclusion**: In arginase deficiency, OPC survival is impacted by elevated guanidino compounds, but not by hyperargininemia, in a creatine-dependent manner & results in pronounced dysmyelination in the early neonatal period. This phenotype can be alleviated by AAV-based gene therapy controlling guanidino compound production. These findings demonstrate the potential impact that successful hepatic gene therapy may have on the developing brain in arginase deficiency due to the better understanding of the underlying disease pathology.



124 Enhanced Enzyme Uptake and Glycogen Clearance in Muscles and CNS of Pompe Mice Administered AAV9 Expressing Novel Engineered GAA

Waijiao Cai[1], Nick Marze[2], Erin Lopez[1], Lucy S. Marie[1], Ilya Tikh[2], Susan A. Benard[2], Vincent Chihyi Yu[2], Michael St. Andre[1], Jane M. Owens[1], Loujin Song[3], Laura Monarski[4], Shambhunath Choudhary[5], Sagar Nisraiyya[1], Bin Li[1], Arpana Khatri[1], Marla Bazile[1], Crystal OH[1], Kruti Patel[1], Jatin Narula[2], Ramesh C. Kovi[6], John Jakubczak[7], Lisa R. Keyes[8], Chen Seng H[1], Clark Pan[1], Sury Somanathan[1]

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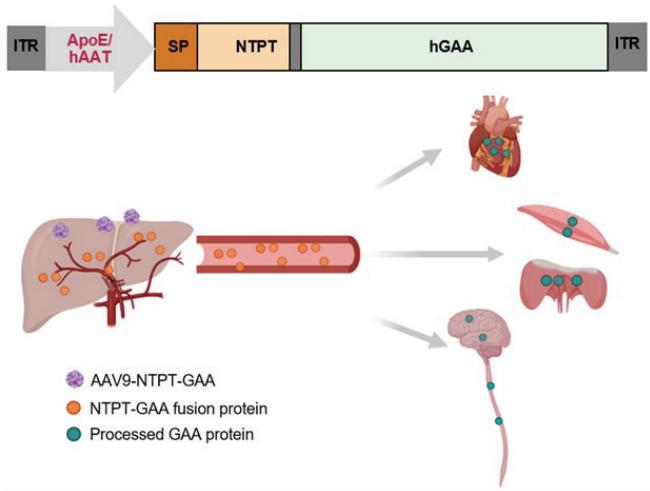
Pompe disease is a life-threatening glycogen storage disorder caused by deficiency of acid-alpha-glycosidase (GAA). The effectiveness of recombinant human acid-alpha-glucosidase (rhGAA) enzyme replacement therapy treatment is limited due to hepatic clearance and insufficient mannose-6-phosphate (M6P) glycans, the latter being required for uptake and lysosomal delivery by M6P receptor (M6PR). Here, we fused an N-terminal peptide tag (NTPT) derived from Insulin-like growth factor-II to hGAA. The chimeric protein NTPT-hGAA also binds to M6PR, albeit at a site that does not require M6P glycans. We first evaluated the NTPT-hGAA and hGAA in a cellular uptake assay by adding increasing amounts of enzyme to cells. Addition of NTPT-hGAA led to high uptake in rat myoblasts and patient fibroblasts; however, hGAA administration, even at the highest level did not result in increased cellular uptake. Systemic administration of AAV expressing NTPT-hGAA to Gaa$^{-/-}$ mice enhanced enzyme uptake, cleared glycogen, corrected autophagic defects in muscle and brain, and restored muscle function. The NTPT was further engineered to reduce undesired binding to Insulin-like growth factor-I receptor (IGF-IR) and insulin receptor (IR). A screen of NTPT variants identified a unique sequence (#44) that demonstrated reduced IGF-1R and IR binding. Signal peptides of NTPT-hGAA and NTPT44-hGAA variants were replaced by an alternate sequence (SP13) to optimize liver secretion. Intravenously administered AAV-SP13-NTPT-hGAA and AAV-SP13-NTPT44-hGAA efficiently reduced muscle, brain, and spinal cord glycogen content. Cynomolgus macaques administered AAV-SP13-NTPT-hGAA and AAV-SP13-NTPT44-hGAA demonstrated dose-dependent liver expression. Vector constructs were well tolerated at the administered dose levels. In an effort to lower the dose and reduce the immunogenicity risk we codon-modified the transgene sequence. These optimized second-generation constructs demonstrated higher NTPT-hGAA expression and activity in a human liver cell line. Systemic administration led to increased serum NTPT-GAA concentration and tissue enzyme uptake in wild type and Gaa$^{-/-}$ mice when compared to non-optimized constructs. Simulation models suggest 3 to 5-fold dose lowering with the new codon-optimized constructs in non-human primates. Taken together, these studies demonstrate an effective

strategy to deliver liver-expressed hGAA to muscle and central nervous system that is glycosylation independent yet minimizes the safety risks associated with off-target receptor engagement.



SP=signal peptide, NTPT=N terminal peptide tag, hGAA=human acid alpha-glucosidase

125 Rescue of a Lethal Murine Model of Methylmalonic Acidemia by AAV Mediated Delivery of PiggyBac Transposase and a Therapeutic Transgene

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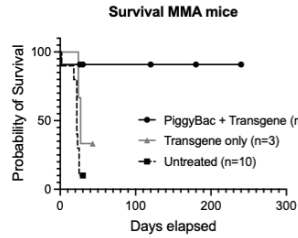
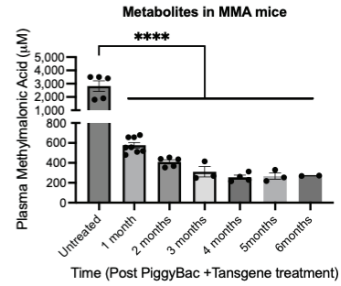
Introduction: Methylmalonic acidemia (MMA) is a severe autosomal recessive metabolic disorder caused most frequently by variants in the methylmalonyl-CoA mutase (MMUT) gene, which encodes for this mitochondrial localized enzyme. MMA results in both acute and chronic multisystemic medical complications that can be fatal. Severely effect patients can undergo liver transplantation to treat the underlying enzymatic defect and the success of this procedure suggests that liver direct gene delivery maybe a viable treatment option. Systemic AAV gene therapy has recently emerged as a promising new therapy for MMA but could be limited by the temporal loss of AAV episomes, a well-recognized limitation of therapy with non-integrating vectors. Here we explore the therapeutic efficacy of a dual AAV transposase gene delivery system to integrate a therapeutic transgene into the hepatocytes of mice using a recently generated mouse model of a lethal form of MMA caused by a MMUT p.R106C variant. Methods: Two AAV vectors were created and pseudoserotyped with an AAV8 capsid: one AAV expresses a therapeutic MMUT transgene under the control of a liver specific promoter, cloned between AAV2-ITRs and internally flanked by transposon-specific terminal repeats (TR), while the second is similarly designed to express a modified PiggyBac transposase but lacks transposon TRs. When both AAVs transduce the same cell, the PiggyBac transposase will mediate a cut-and-paste insertion of the *MMUT* transgene into the genome. Either the transgene only or transgene plus Piggybac vectors were delivered at birth by retroorbital injection, at doses of 5.4e13 vg/kg (transgene) and 4.3e13vgkg (PiggyBac), to MmutR106C/R106C mice and control littermates. Survival, growth, metabolite levels, and transgene expression were assayed.Results: *Mmut*$^{R106C/R106C}$ mice treated with PiggyBac+transgene had significantly ($p<0.01$) increased survival versus both untreated and transgene only treated mice. While rare untreated *Mmut*$^{R106C/R106C}$ mice that survived to weaning were approximately 40% the mass of normal wildtype mice, PiggyBac+transgene treated mice had normal growth, not significantly different than control littermates . Plasma levels of the disease-related biomarker, methylmalonic acid, in PiggyBac+transgene treated mice were significantly lower than in untreated mice at all time points measured out to 6 months posttreatment ($p<0.001$). Lastly, in situ hybridization to detect transgene expression in the livers harvested from PiggyBac+transgene treated mice revealed large numbers of hepatocytes expressing *MMUT* mRNA one month after treatment, with abundant positive staining hepatocytes, widely distributed throughout the liver.Conclusions: AAV mediated gene editing using a transposase to enable hepatic integration of a therapeutic transgene enables the permanent correction of the clinical manifestations of MMA in a disease model with a severe and lethal phenotype. The modular design of the vectors we have developed should enable the rapid assessment of other transposable elements for therapeutic benefits in the treatment of MMA, and by extension, other disorders of intermediary metabolism where early hepatic correction is beneficial, and stable transgene expression is required.





126 New Mouse Models of, and AAV9 Gene Therapeutics to Treat, Isolated Methylmalonic Acidemia, Cobalamin B (*cblB*) Type

Eun-Young Choi, Karenna Choi, Lina Li, Charles P. Venditti
MMB, OARS, NHGRI, NIH, Bethesda, MD

Introduction: Cobalamin B-type (*cblB*) methylmalonic acidemia (MMA) is an rare and severe autosomal recessive inborn error caused by pathogenic variants in the MMAB gene, which encodes the enzyme ATP:cob(I)alamin adenosyltransferase (ATR) and catalyzes the synthesis 5'- deoxyadenosylcobalamin (AdoCbl), the cofactor for methylmalonyl-CoA mutase (MMUT). MMUT and MMAB deficiencies are clinically similar, recalcitrant to medical management, and treated using elective liver (LT) and liver-kidney transplantation (LKT). There are no animal models or gene therapies that have been developed for MMAB deficiency, a Platform Vector Gene Therapy (PaVe-GT) indication. **Methods:** We assessed the correlations between patient mutations, clinical phenotypes and treatment with transplantation in a cohort of N=17 subjects with MMAB deficiency ascertained via a natural history study of MMA (NCT00078078). Two severe mutations, the orthologue of a missense mutation recurrently noted in those who received LT/LKT (p.Arg186Trp), and another that removed exons 3-7, were engineered into the Mmab locus. In addition, a germline

rescue transgene, designed to restore expression of MMAB in the skeletal muscle under the control of the murine muscle creatine kinase (MCK) promoter, was knocked into the ROSA26 locus ($Tg^{MCK\text{-}Mmab}$), and used to generate a hypomorphic model ($Mmab^{R180W/R180W}$; $Tg^{MCK\text{-}Mmab}$). AAV vectors to express *MMAB* under the control of the EF1L or EF1s promoters, in either the single strand (ss) or self-complementary (sc) genome configurations respectively, were pseudoserotyped with an AAV9 capsid, and assayed for in vivo efficacy. **Results:** $Mmab^{\Delta 3\text{-}7/\Delta 3\text{-}7}$ and $Mmab^{R180W/R180W}$ mice displayed lethality and massively increased plasma [MMA] levels (1150 +/- 177 uM, 360x WT), very much like patients with severe forms of *cblB* MMA. $Mmab^{R180W/R180W}$;$Tg^{MCK\text{-}Mmab}$ mice survived until weaning but manifested pronounced MMAemia (520+/- 79 uM, 200x WT), growth retardation, increased FGF21, and decreased ^{13}C propionate oxidation. In a proof of concept study, neonatal treatment of $Mmab^{\Delta 3\text{-}7/\Delta 3\text{-}7}$ mice (n=6) with the scAAV9 EF1s MMAB vector, systemically delivered at a dose of 1e11 GC/pup on DOL1, resulted in complete rescue from lethality, and full phenotypic correction persisting > 100 days (Figure 1). $Mmab^{R180W/R180W}$; $Tg^{MCK\text{-}Mmab}$ mice treated with either vectors as young adults (n=6), given at a dose of 1e13 GC/kg, produced equally robust responses by 2 weeks, with a 38x reduction (12.9 +/- 3.7 uM) of the circulating plasma [MMA], restoration of ^{13}C propionate oxidation to wild type levels, and transgene expression in the liver (Figure 2) and kidney (Figure 3) exceeding WT MMAB levels. **Conclusions:** In this work, we have developed a series of clinically relevant MMAB animal models and AAV9 vectors that mediate robust hepatorenal transgene expression after systemic delivery. These mouse models can be used for neonatal and adult proof of concept therapeutic studies, including dose finding and pharm-tox, and will be used to rapidly enable a new class of AAV9 gene therapeutics to treat patients with MMAB deficiency, a severe form of MMA.

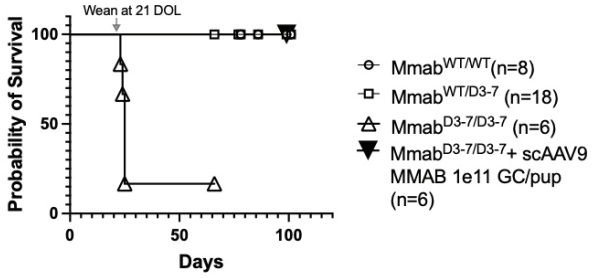


Figure 1

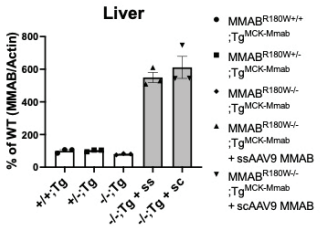


Figure 2

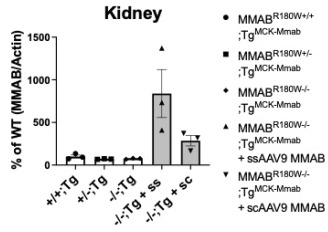


Figure 3

127 Preclinical Proof-of-Concept: A Novel Hybrid Gene Therapy Approach to Treat Severe Early-Onset Ornithine Transcarbamylase Deficiency

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Ornithine Transcarbamylase deficiency (OTCD) is an X-linked urea cycle disorder that prevents the breakdown and excretion of ammonia; this allows ammonia to rise to toxic levels and affect the central nervous system leading to coma, seizures, brain damage, and death. For young, early-onset patients, the disease burden is severe and without treatment can be fatal. Liver transplant is the only current corrective option, as other interventions do not enable survival past childhood for most patients. Thus, there is an urgent unmet need for efficient therapies to rescue the disease burden by intervening early in life. Gene therapy approaches utilizing recombinant adeno-associated virus (rAAV) are likely to be ineffective when used early in life due to the expected dilution of the therapeutic transgene during cell division commensurate with liver growth. Currently, re-dosing of AAV is not feasible due to the strong inherent immunity against AAV capsids. We have developed a gene therapy platform utilizing the super piggyBac® DNA insertion system that enables integration of the therapeutic human OTC gene into the genome. Genomic integration of the OTC gene enables it to be maintained during cell division, potentially enabling durability of the therapeutic effect throughout life. The system entails two components: 1) a piggyBac transposon comprising the human OTC gene under a weak liver-specific promoter and formulated as a liver-tropic AAV(AAV-hOTC) and 2) an mRNA coding for the super piggyBac transposase (SPB) formulated as a liver-directed lipid nanoparticle (LNP-SPB). Severe OTCD was modelled in the hypomorphic OTC-deficient mice (spf^{ash}), using an AAV-delivered shRNA to knock down residual OTC activity. Mice were treated on day=1 of life by intravenous administration of the hOTC-AAV and LNP-SPB. As a negative control, some mice were administered the AAV-hOTC alone or AAV-hOTC with an LNP containing an mRNA for the catalytically deficient form of SPB that is unable to mediate genomic integration of the hOTC transgene. Mice were administered the shRNA construct forty days post-treatment, which rapidly induced hyperammonemia and death within 10 days. Treatment with the AAV-hOTC alone or AAV-hOTC combined with the catalytically deficient SPB LNP did not meaningful extend the survival of these mice even at high AAV dose (5E13 vg/kg), and immunohistochemical analysis of the liver showed only few and scattered (<3%) hepatocytes expressing the hOTC transgene. In contrast, mice treated with the AAV-hOTC and LNP-SPB exhibited full survival at AAV doses as low as 5E12 vg/kg, coupled with normalization of plasma ammonia and urinary orotic acid to wild type levels. Immunostaining demonstrated a substantial (>40%) proportion of hepatocytes expressing the hOTC gene, which persisted well into adulthood. Integration analysis showed low integrated copy numbers (1-3 hOTC per diploid genome) in

the liver, and the frequency of integrations was proportional to the dose of LNP-SPB. No clinically significant elevations in liver enzymes were observed in mice or non-human primates at any of the evaluated doses. Serum pro-inflammatory cytokines were transiently elevated and proportional to LNP-SPB dose. Both the AAV and LNP exhibited strong liver tropism, with no detection in brain or gonad. These data demonstrate the correction of severe OTCD, entailing complete survival and normalization of biomarkers of urea cycle function, following a single administration of our integrating gene therapy administered on day 1 of life and supports subsequent development toward evaluation in human patients.

128 Long-Term Safety and Efficacy of DTX301 in Adults with Late-Onset Ornithine Transcarbamylase (OTC) Deficiency: A Phase 1/2 Trial

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Background: OTC deficiency is an X-linked urea cycle disorder resulting in life-long risk of episodic hyperammonemia that can lead to coma or death, even with ongoing treatment with ammonia scavenger medication and dietary protein restriction. DTX301 is an AAV8 vector containing the OTC transgene being investigated for treatment of OTC deficiency. We report the long-term follow up (LTFU) of 11 adults with late-onset OTC deficiency who received a single IV infusion of DTX301 in the Phase 1/2 trial (NCT02991144). **Methods:** The initial Phase 1/2 study lasted 52 weeks; all participants will be followed thereafter semi-annually for up to an additional 5 years. Complete responders discontinued all ammonia-scavenging drugs and protein-restricted diet. Responders had ≥50% reduction in medications and dietary protein restriction. Data cutoff was 09 Dec 2022. **Results:** Currently, no treatment-related serious AEs, dose-limiting toxicities, or infusion-related events have been reported. One patient experienced a hyperammonemic crisis, and two patients experienced mild ALT elevations during LTFU (all considered unrelated to DTX301). As of cutoff, 7/11 patients have shown a continued response to DTX301 from 2 to now over 5 years after dosing; 4 patients were complete responders, and 3 patients were responders (**Table**). Plasma glutamine levels showed considerable individual variability but were generally lower for complete responders and responders. Citrulline levels were in the normal range and generally stable for all groups. Overall mean plasma ammonia area under the curve $(AUC)_{0\text{-}24}$ showed improvement over time with variability. Values were more consistently in the normal range (< 1440 µmol*h/L) for complete responders and responders. **Conclusions:** DTX301 continues to suggest a manageable safety profile and encouraging evidence of durable efficacy with the longest treated complete responder exhibiting efficacy for over 5 years. Patients in this study will continue to be followed. The Phase 3 trial (NCT05345171) is currently recruiting.

Responder status		AUC (µmol*h/L)			Glutamine (µmol/L)		Citrulline (µmol/L)		Time since dosing (yrs)
W52	Last Visit	Baseline	W52	Last Visit	Baseline	W52	Baseline	W52	
Complete Responder	Complete Responder	982	1406	1366	571	904	20	25	5.4
Complete Responder	Complete Responder	1090	899	856	469	661	39	23	4.9
Complete Responder	Complete Responder	NA	1024	1846	681	661	17	17	4.3
Complete Responder	Complete Responder	2606	1086	NA	1125	609	20	16	2.1
Responder	Responder	4445	3850	NA	528	857	14	79	3.6
Responder	Responder	NA	1601	1132	557	690	86	34	3.4
No response	Responder	3748	NA	1258	1164	857	49	46	4.8
No Response	No Response	3501	1844	3008	768	815	13	12	5.4
No Response	No Response	1013	1101	1502	540	534	12	13	5.2
No Response	No Response	1623	1040	1206	1007	1231	20	22	4.8
No Response	No Response	1063	1864	NA	1108	1007	13	10	2.4

129 Single AAV Vector Gene Therapy with a Mini-GDE Transgene Corrects Muscle Impairment in Mouse, Rat and Human Cellular Models of Glycogen Storage Disease Type III

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Glycogen storage disease type III (GSDIII) is a rare inborn error of metabolism affecting liver, skeletal muscle, and heart due to mutations of the *AGL* gene encoding for the glycogen debranching enzyme (GDE). The 4.6 kb GDE cDNA represents a technical challenge toward the development of a single *adeno-associated virus* (AAV) vector strategy, since an expression cassette containing the full-length GDE cDNA is largely oversized. Dual vectors or the use of bacterial orthologs of GDE were proposed to overcome this size limitation. However, the clinical translation of these approaches is complicated by possible immune responses and the higher doses required. Here we developed a single rAAV-based gene therapy strategy for GSDIII using a truncated GDE to correct skeletal muscles and heart impairment in $Agl^{-/-}$ mice and rats and to decrease glycogen content in skeletal myotubes differentiated from $AGL^{-/-}$ human pluripotent stem cells (iPSC). Using molecular modeling, the 3D structure of GDE and considering the positions of known *AGL* missense mutations, we identified the GDE N-terminal as the most promising region to reduce the size of the enzyme while retaining its activity. Particularly, the ΔNter2 mutant showed similar glycogen clearance *in vivo* to the full-length GDE. Moreover, this mutant size allowed encapsidation with strikingly improved rAAV vector yields and quality. Intravenous injection of ΔNter2-expressing rAAV vectors in adult $Agl^{-/-}$ mice and rats allowed complete glycogen clearance in muscles and heart and normalization of histology features

three months after injection. It also allowed complete reversal of the muscle strength impairment in *Agl*$^{-/-}$ mice. Finally, transduction of AGL$^{-/-}$ iPSC-derived muscle cells with ΔNter2-expressing rAAV vectors resulted in almost complete glycogen clearance with no overt toxicity compared to mock-transduced cells. In conclusion, our results show for the first time the ability of a functional truncated GDE transgene to correct the muscle and heart phenotype in multiple models of GSDIII, supporting its clinical translation in GSDIII patients

Gene Therapy Approaches for Muscle and Skeletal Diseases

130 AAVrh74.tMCK.hBAG3 Gene Therapy Improves Function and Decreases MYOT Aggregate Formation in TgT57I Mice

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Myofibrillar myopathies (MFMs) are a group of protein aggregate diseases, defined histologically by abnormal protein aggregations and myofibrillar disintegration. Myotilinopathy, also called limb-girdle muscular dystrophy type 1A (LGMD1A), belongs to the distal MFM group and is characterized by initial weakness of proximal girdle muscles. Mutations in myotilin gene have been associated with myotilinopathy and the underlying pathology was suggested to be toxic gain-of-function leading to myotilin-positive protein aggregates as well as a loss-of-function that impairs the integrity of myofibrils. Degradation of non-native proteins can be facilitated by Ubiquitin-proteasome system, however when this pathway is overloaded under pathophysiological conditions, the protein quality control system leans on the autophagy-lysosome pathway (ALP) to mediate degradation of aggregates. BCL2-associated athanogene 3 (BAG3) protein facilitates aggresome formation and initiates this macroautophagic pathway. In this study, we assessed our strategy of reducing the aggregate burden in the muscle by overexpressing the BAG3 in a mouse model for LGMD1A, carrying a human myotilin gene with T57I mutation. Ten male TgT57I mice received 3x10^{12} vg total dose of AAVrh74.tMCK.BAG3, via tail vein injection, aged 4-8 weeks, and eight sex and aged-matched TgT57I mice served as untreated (UT) control. Mice were tested for rotarod, treadmill, grip strength and *in vivo* muscle contractility, which included maximum twitch and tetanic responses at the endpoint, around 8 months post gene delivery. The BAG3-treated cohort demonstrated increased rotarod duration by 43.2% (BAG3, 39.54 ± 1.33 sec; UT, 27.6 ± 1.77 sec; p=0.0001) and running distance on treadmill by 70.5% (BAG3, 156.03 ± 13.93 m; UT, 91.53 ± 13.35 m; p=0.0048), compared to UT cohort. Grip-strength values of treated cohort improved by 38.7% (BAG3, 0.102 ± 0.008 kg; UT, 0.074 ± 0.004 kg; p=0.006). The *in vivo* muscle contractility assay showed that muscle strength was increased significantly for maximum tetanic response (BAG3, 6.26 ± 0.44 mN*m; UT, 5.19 ± 0.23 mN*m; p=0.047), while the increase was not significant for maximum twitch response. Myotilin aggregate burden was quantified using immunofluorescence technique on 4 randomly selected representative images at 20x-magnification from quadriceps muscles of BAG3-treated (n=10) and UT (n=8) cohorts. The number of myotilin positive aggregates per unit area was decreased significantly in the treated group, compared to untreated counterparts (BAG3, 466.1 ± 31.3 /mm^2 vs. UT, 585.6 ± 47.9 /mm^2; p=0.0007). Aggregate size distribution analysis revealed a reduction of numbers in all aggregate sizes with treatment, demonstrating significance for aggregate sizes up to 10 μm in diameter. We also noted a decrease in mean aggregate fluorescence intensity with treatment, without reaching significance level. In conclusion, systemic BAG3 gene therapy in a mouse model for LGMD1A have significantly improved function and maximum tetanic response compared to UT cohort. Efficacy of AAVrh74.BAG3 was supported by significantly decreased aggregate number in the treated cohort as proof of principle that our strategy of reducing the aggregate burden in the muscle, by overexpressing BAG3, may be used in treatment for protein aggregate myopathies.

131 Toxicology, Pharmacokinetics and Biodistribution of a PATrOL™-Enabled Investigational Genetic Therapy for Myotonic Dystrophy, Type 1

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Patients with myotonic dystrophy, type 1 (DM1) suffer from cognitive deficits and muscle pathologies caused by a trinucleotide repeat expansion on one allele of the *DMPK* gene. This mutation, when transcribed, results in a toxic mRNA that sequesters splice proteins resulting in broad-based splice dysregulation and the generalized signs and symptoms of the disease. In order to address this disease, we utilized our peptide nucleic acid-based platform, PATrOL™, to develop both a novel PNA-based pharmacophore and a novel delivery technology that allows systemic routes of administration. Previously, we have described the ability of a DM1 lead candidate to correct the pathogenic lesion through knock-down of the human *DMPK* transcript and correction of downstream splicing defects in both fibroblasts and myoblast cultures derived from DM1 patients. We subsequently determined the EC50 to be 30nM, independent of the trinucleotide repeat expansion length. We have reported molecular and functional pharmacologic activity in the HSA^{LR} transgenic mouse model where treatment with our lead candidate rapidly reduced mutant transcript levels, corrected splicing defects, and rescued myotonia. This pharmacophore is delivered via our novel delivery technology. Previously we have demonstrated pharmacological activity of various pharmacophores in both brain and muscle after systemic routes of administration including both intravenous and subcutaneous routes. In addition, a radiolabeled biodistribution study with the delivery module after intravenously administration in nonhuman primates showed distribution to brain, muscle, and heart, and all major organs affected in DM1 patients. Additional three-month PK studies in NHP

have been completed and illustrate tissue-specific elimination rates that facilitate the design of multi-dose regimens for the Phase 1/2 trials. This data will be presented. Recently we have completed exploratory toxicology studies in rodents and non-human primates (NHPs) and have determined the single dose IV maximum tolerated doses (MTDs) based upon histology to be less than 29 mg/kg. The toxicology signal is seen in the kidney and is exemplified by tubular degeneration and followed presumably after the C_{max} with regeneration of tubules noted. Our lead candidate displayed no abnormal blood chemistries up to and including the MTD, and no test-article related immunogenicity in NHPs. A three-month pharmacokinetic (PK) study was completed in NHPs and based upon the EC50 value for patient-derived myotubes, we anticipate a human effective dose of <2.0 mg/kg. This data will be presented.

132 Management Of Patients Following Investigational Delandistrogene Moxeparvovec Gene Therapy For Duchenne Muscular Dystrophy: Delphi Panel Consensus Considerations Based on Clinical Trial Experience

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Background/Objectives: Delandistrogene moxeparvovec is an investigational rAAVrh74-based gene transfer therapy for the treatment of Duchenne muscular dystrophy (DMD) by expression of SRP-9001 dystrophin protein. An integrated analysis of 3 ongoing clinical trials for individuals with DMD (N=85) reported collective safety outcomes following the single intravenous infusion of delandistrogene moxeparvovec. Most treatment-related treatment-emergent adverse events (TEAEs) occurred within 90 days of treatment. A Delphi panel convened to formulate expert consensus recommendations for management of select TEAEs that require urgent medical intervention, including vomiting, acute liver injury (ALI), myocarditis, and immune-mediated myositis (IMM). **Methods:** A panel of 12 US-based neuromuscular, hepatology, cardiology, and gene therapy experts utilized a modified Delphi process to reach consensus guidance on TEAE management. Consensus was defined as a majority of experts either agreeing or disagreeing (5-point Likert scale). Surveys were developed based on systematic literature review, data on file, clinical trial experience, and expert panel responses. All experts completed two rounds of virtual surveys followed by a live meeting. **Results:** An as-needed antiemetic was recommended for vomiting. Initial mild elevations of liver laboratory tests should be assessed with repeated labs and close monitoring. If ALI is diagnosed, considerations for additional diagnostic testing, baseline corticosteroid dose adjustment, and hospitalization should be based on timing of onset and severity of symptoms. For patients with elevations in troponin I, considerations should be based on the severity of the elevation and presence of symptoms, and include corticosteroid optimization, repeated labs, electrocardiogram, echocardiogram, and cardiac MRI. Experts agreed that for suspected symptomatic IMM, emergent evaluation by physical exam, labs, and additional diagnostics would be required. In addition to hospitalization and optimization of corticosteroids, non-steroidal immunosuppressive therapy could be considered depending on the clinical scenario. **Conclusions**: The Delphi recommendations offer initial guidance that can be considered by clinicians for management of selected TEAEs following delandistrogene moxeparvovec therapy.

134 Lentivirus-Based Hematopoietic Stem/Progenitor Cell Therapy Provides Safe and Long-Term Treatment of Hypophosphatasia in Murine Models

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Hypophosphatasia (HPP) is a multi-system metabolic disorder characterized by defective bone mineralization and skeletal abnormalities, as well as complications such as seizure, muscle weakness and respiratory failure. It is caused by loss of function mutations of the tissue-nonspecific isoenzyme of alkaline phosphatase (TNALP). HPP has a wide range of clinical manifestations and can affect patients of all ages with devastating outcomes at any stage of life. Severe forms of HPP in infants and young children is life-threatening. Currently, enzyme replacement therapy with asfotase alfa is the only treatment that enhances survival and improves bone mineralization. However, it does not cure the disease and patients must remain on the therapy for the rest of their lives as discontinuation of asfotase alfa leads to reappearance of bone hypomineralization. Here we report successful development of a hematopoietic stem/progenitor cell (HSPC) based lentiviral gene therapy to provide lifelong treatment for HPP. In humanized mice, we demonstrated that human HSPCs modified with lentivirus vectors (LVVs) expressing soluble forms of TNALP allow safe and successful engraftment of HSPCs and differentiation into multiple hematopoietic lineages. Using a murine model of severe HPP, we demonstrated that in TNALP-/- neonates transplanted with TNALP-expressing LVV-modified HSPCs, correction of plasma ALP level was fast and durable and significantly improved life span. X-ray and micro-CT analysis

showed significantly improved bone structure and mineralization in treated mice. Open field analysis and grip strength tests also demonstrated significantly improved musculoskeletal activities, to levels similar to wildtype mice. Importantly, we observed successful long-term engraftment of the LVV-modified HSPCs that supported differentiation into the expected hematopoietic lineages, resulting in continued correction of plasma ALP level and bone structure over the 11 month study duration, after a single transplantation. Our study provides critical insights into treating bone metabolic disorders with an HSPC-based gene therapy and demonstrates that this approach has the potential to offer a safe and permanent treatment for HPP.

135 AUF1 Gene Therapy for Duchenne Muscular Dystrophy Increases Durable Endogenous Utrophin Expression, Muscle Regeneration and Muscle Function Performance in Pre-Clinical Animal Studies

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Duchenne Muscular Dystrophy (DMD) is one of the most severe disorders of muscle degeneration, caused by mutations in the *DMD* gene encoding dystrophin. Dystrophin is central to the oligomeric dystrophin-associated protein complex (DAPC) that links skeletal and cardiac muscle cell actin to extracellular laminin, stabilizing the muscle cell membrane known as the sarcolemma. The absence of dystrophin leads to progressive muscle degeneration, chronic inflammation, and fibrosis. Because the dystrophin gene is too large to package in AAV vectors, gene therapies for DMD express subsets of dystrophin domains to generate truncated "microdystrophins" (μDys). While efficacy has been shown in animal and human clinical trials, issues remain, particularly an inability to rescue muscle stem cell (satellite cell) exhaustion and low regenerative capacity of DMD muscle and loss of slow-type, oxidative myofibers. Therefore, there remains an urgent need for approaches that address the full suite of pathophysiological features in DMD including loss of muscle strength, endurance regenerative capacity, myogenesis program dysfunction and restoration of the satellite cell niche population. AU-rich mRNA binding factor 1 (AUF1) is an RNA binding protein that binds repeated AU-rich elements (AREs) located in the 3' untranslated region of approximately 3% of mRNAs. AREs target mRNAs for either rapid degradation or stabilization, and/or increases ARE-mRNA translation, largely controlled by AUF1. We showed that many key regulators of muscle satellite cell activation and maintenance, myogenesis and oxidative metabolism are encoded by ARE-mRNAs regulated by AUF1, including PGC1α. Moreover, we previously reported that systemic AUF1 gene therapy strongly increases skeletal muscle expression of the dystrophin homolog utrophin, which can functionally substitute for dystrophin in the DAPC, by binding to and stabilizing its mRNA. Thus, AUF1 directly regulates skeletal muscle maintenance, regeneration, mitochondrial biogenesis, and myofiber integrity, providing strong rationale for AUF1 gene augmentation as a therapy for DMD. As proof of principle, one-month-old *mdx* DMD mice were administered i.v. AAV8.tMCK.AUF1. After 3 and 6 months of gene therapy, treated mice demonstrated significantly increased physical endurance and strength by all testing parameters compared to *mdx* controls. Remarkably, AUF1 gene therapy ameliorated the diaphragm and skeletal muscle dystrophic phenotype, reduced myofiber centro-nucleation, embryonic myosin heavy chain (eMHC) expression, and elevated serum creatine kinase. AUF1 increased utrophin, leading to an increase in DAPC protein expression and improvement of diaphragm and limb skeletal muscle integrity and morphology. Because AUF1 acts through a distinct mechanism from μDys, we tested a tandem AAV8.AUF1/AAV8.μDys approach. μDys combined with low level AUF1 demonstrated superior muscle function strength and endurance compared to AUF1 or micro-dystrophin alone (91% of WT grip strength, 70% of WT time to exhaustion) for age and sex matched mice. Furthermore, the μDys/AUF1 combination demonstrated superior muscle morphology, myofiber size, near normal satellite cell numbers, protection of muscle from atrophy following muscle injury and durable increased exercise endurance. Altogether, these results are compelling evidence that AUF1 mono- or μDys combination gene therapy may offer an alternate and superior approach in the treatment of DMD.

136 Intra-Articular Delivery of AAV Vectors Encoding Immune Checkpoint Protein as a Novel Treatment for Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by skewed and dysregulated immune responses that affect multiple organs, particularly the joints. Currently, there is no cure for RA, and patients are usually treated with one or more disease-modifying anti-rheumatic drugs (DMARDs) and/or biologics, such as cytokine inhibitors, to inhibit immune and inflammatory responses. However, less than 30% of patients have robust responses to these drugs, and achieve long-term remission. It is well known that immune cells, particularly T cells and macrophages, infiltrate the joints in inflammatory arthritis.Therefore, targeting immune cells may represent a more ideal strategy to improve arthritis treatment outcomes when compared to single cytokine. Immune checkpoints are known to regulate immune cells by balancing the co-stimulatory and co-inhibitory signals. In this study, we explored the therapeutic role of immune checkpoint protein PD-L1 in RA after intra-articular administration of adeno-associated virus (AAV) vector encoding human PD-L1. In a mouse model with the collagen induced arthritis (CIA). 5 x 10^9 vg AAV5/PD-L1 vectors encoding wild type PD-L1 with the transmembrane domain were injected into the knee joints of male DBA/1J mice at age of 7-8 weeks on the day 0 of priming immunization of collagen II or day 21 of booster post primary immunization. On day 49 post primary immunization, we found that mice treated with AAV5/PD-L1 vectors had experienced a 4-fold decrease in joint swelling and a 2.5-fold lower histopathological score compared to the control mice injected with AAV5/luc vectors. The infiltration of T cells and macrophages was remarkably lower in the joints of CIA mice that had received AAV5/PD-L1 vectors ($P<0.05$). Levels of pro-inflammatory cytokines, including IL-1, IL-6, IL-17 and TNFα, were

also 2-4 fold lower in the AAV5/PD-L1 treated joints than that in the control joints (P<0.05). Strong PD-L1 expression was detected in AAV transduced joints but no PD-L1 protein expression was detected in other tissues. High AAV genomes were detected in the AAV5/PD-L1 treated knees with ~ 1.3 viral copy numbers per diploid genome and in the liver at levels of about 0.008 viral copy numbers per diploid genome. Furthermore, the administration of AAV5/PD-L1 vectors into the joints of RA mice didn't impact the antibody titers to type II collagen and the cytokine levels in the serums. Similar to the results of AAV5/PD-L1 vector administration on day 0, decreased joint swelling and lower histopathological damage were observed in joints treated with AAV5/PD-L1 vectors on day 21. To further enhance the PD-L1 efficiency, three soluble PD-L1 variants with different transmembrane domain knockouts (shPD-L1, hPD-L1, secPD-L1) were constructed. After transfection into 293 cells, we found that shPD-L1 was able to induce much higher transgene expression in both the supernatant and the cell lysate than other two variants; total shPD-L1 protein expression in the HEK-293 cells was 7 fold higher than that of wildtype PD-L1. Next, we compared the efficiencies of wildtype PD-L1 and shPD-L1 to inhibit arthritis in CIA mice. After intra-articular injection of different doses of AAV vectors at day 0, the similar arthritis improvement was achieved with the minimum dose for AAV5/PD-L1 at a dose of 5×10^7vg and for AAV5/shPD-L1 at the dose of 5×10^6 vg, indicating that shPD-L1 has a more potent effect than wild type PD-L1. The results from this study demonstrate that AAV mediated PD-L1 gene delivery into the joints is able to prevent the development and block the progression of arthritis in CIA mice without impacting systemic immune responses, and provides a novel strategy to effectively treat chronic inflammatory joint diseases by interference with immune checkpoint pathways using local AAV gene therapy.

Understanding and Modeling Immune Responses to Gene Therapy and Vaccines

137 High-Dose AAV Toxicity in Mice: Serotype-Dependent Hepatocellular Damage and Complement Deposition and Activation

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Clinical success has been achieved with adeno-associated virus (AAV) gene therapies that result in secreted proteins or are confined to small regions of organs. However, for disease settings in which gene delivery and expression over a broad area of tissue are required (e.g., muscular dystrophy), the larger delivery burden has necessitated high systemically administered doses of AAV to achieve therapeutic levels of protein expression. Such high-dose AAV regimens have led to unanticipated toxicities in clinical trials, most often including thrombocytopenia, liver damage, and complement activation. To ameliorate these toxicities, a better preclinical animal model that is predictive of high-dose vector toxicity is needed. Here, we systemically administered high doses of AAV expressing enhanced green fluorescent protein (eGFP), while varying capsid serotypes, doses, and DNA content. In wild-type C57BL/6J mice receiving 3×10^{14} genome copies (GC)/kg of AAV9, we observed elevated levels of C3b (the cleavage product of complement factor C3 upon activation) in the plasma beginning at day 1 and peaking at day 3. This systemic elevation of C3b corresponded in time with histological findings of the terminal membrane attack complex of the complement cascade, C5b-9, in treated livers. Histopathological analysis of these livers at day 3 revealed single-cell hepatocellular necrosis, consistent with elevated serum transaminase levels peaking at day 3 post vector administration. These toxicities were more pronounced when mice received a supraphysiologic dose of AAV9.eGFP at 7×10^{14} GC/kg, where a mild thrombocytopenia arose at day 7. Importantly, dosing mice with AAV9.eGFP at 10^{14} or 5×10^{13} GC/kg avoided most of the toxicities observed at doses above 3×10^{14}GC/kg, establishing an apparent dose threshold. Interestingly, AAV8 and AAVrh74 vectors expressing eGFP administered at 3×10^{14} GC/kg in C57BL/6J mice elicited minimal to no toxicity. AAV8- and AAVrh74-treated mice did not display elevated liver transaminases and exhibited only infrequent mild hepatocellular necrosis on histopathological analysis. There was evidence of complement activation (C3b) and deposition (C5b-9) in the liver, but to a much lesser extent than what was observed in AAV9-dosed mice. Next, we interrogated which elements were responsible for the observed toxicity in high-dose AAV9.eGFP administration. We first generated empty AAV9 vectors with no packaged DNA to assess whether 3×10^{14} GC/kg equivalent particles administered systemically to C57BL/6J mice would elicit toxicity. Eliminating the packaged DNA prevented complement activation and deposition and led to a marked delay and reduction in transaminases and single-cell hepatocellular necrosis. This result confirmed that both the capsid serotype and the presence of packaged DNA contribute to the observed systemic toxicities in wild-type mice. We subsequently administered AAV9.eGFP at 3×10^{14} GC/kg in mice lacking C3 (C57BL/6J background strain), a key node in the proteolytic complement cascade. Like empty AAV9 in wild-type mice, a high dose of AAV9 in C3-knockout mice resulted in the loss of complement activation and deposition, and a delayed occurrence of transaminitis and single-cell necrosis. In summary, we observed high-dose AAV toxicity in mice in a serotype-specific manner, with DNA-containing AAV9 eliciting the largest array of toxicological findings. Observing these clinical toxicities in a mouse model will help inform future efforts in pre-clinical toxicity models of high dose AAV.

138 Effectiveness of Engineering AAV Vector Genomes to Evade TLR9 Signaling for Prevention of CD8$^+$ T Cell Responses in Muscle Gene Transfer is Vector Dose Dependent

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Gene therapy using adeno-associated viral (AAV) vectors is being widely evaluated in clinical trials for the treatment of neuromuscular disorders. However, immune responses to vector and transgene product are a major concern. It has been established that recognition of pathogen associated molecular patterns by innate immune sensors drives the activation of CD8$^+$ T cells. For instance, Toll-like receptor

(TLR) 9 senses unmethylated CpG motifs in AAV genomes (one of the hallmarks of viral DNA), resulting in type I IFN mediated activation of anti-viral response. However, our recent studies show that similar to WT mice, TLR9$^{-/-}$ mice elicit transgene product specific CD8^{+} T cell response following intramuscular (IM) administration of AAV1 vector at a dose of 2x10^{11} vg/mouse. Interestingly, blockade of IL-1 signaling in TLR9$^{-/-}$ mice substantially reduced these CD8^{+} T cell responses (*24th ASGCT Annual Meeting abstract# 70*). In the present study we evaluated how engineering of AAV genetic pay load (either depletion of CpG motifs or incorporation of a TLR9 inhibitory sequence) effects transgene product specific CD8^{+} T cell response. We also tested the effect of combining IL-1 blockade with TLR9 inhibitory sequence in AAV cassette on these CD8^{+} T cell response. Further, we examined the role of RNA (TLR3-TRIF pathway) and cytoplasmic DNA (cGAS-STING pathway) sensing in mediating transgene product specific CD8^{+} T cell responses. In order to minimize or antagonize TLR9 activation, AAV vectors expressing ovalbumin (OVA) containing either depleted CpG motifs (AAV1-OVA-CpG^{-}) or TLR9-inhitbitory sequence (AAV1-OVA-TLR9i) were produced. Different groups of C57BL/6 WT mice (n=5) were injected with doses of either 2x10^{10} vg (low) or 2x10^{11} vg (high) of these vectors via IM route. For IL-1 blockade studies, C57BL/6 WT mice (n=5) received the high vector dose and were treated with a combined regimen of anti-IL-1α and anti-IL-1β (2x/wk for 5 wks). To study the role of RNA and cytoplasmic DNA sensing C57BL/6 WT mice or knockout of various innate sensors on C57BL/6 background (n=5) were injected with low- or high-dose of AAV1-OVA vector. All groups of mice were bled weekly and OVA-specific CD8^{+} T cell frequencies were monitored using MHC I tetramer (H2-K^{b} -SIINFEKL) for up to 6 weeks. At the low dose, mice injected with AAV1-OVA-TLR9i failed to generate transgene product specific T cell response (P<0.0001). Similarly, lower levels of tetramer+ cells were observed in mice injected with AAV1-OVA-CpG^{-} as compared to AAV1-OVA injected mice (P=0.0051). However, at the high dose, AAV1-OVA-CpG^{-} injected mice had tetramer^{+} cells comparable to mice injected with AAV1-OVA vector. A small, non-significant reduction of tetramer^{+} cells was observed in AAV1-OVA-TLR9i injected mice. Combining AAV1-OVA-TLR9i vector administration with IL-1 blockade significantly reduced the CD8^{+} T cell response but did not eliminate it completely. These results led us to further interrogate other innate sensors such as RNA and cytoplasmic DNA sensors for their role in these CD8^{+} T cell responses. In comparison to WT mice a non-significant reduction in tetramer^{+} cells was observed in STING$^{-/-}$ mice whereas cGAS$^{-/-}$ mice had significantly higher numbers of tetramer^{+} cells, arguing against a substantial effect of cytoplasmic DNA sensing. TRIF$^{-/-}$ and TLR3$^{-/-}$ mice showed reductions in the response that was again vector dose dependent, similar to mice deficient in TLR9 or IL-1 signaling. In summary, our data reveal that engineering of the vector genome to minimize TLR9 activation can be effective in preventing CD8^{+} T cell responses to the transgene product at lower vector doses but may require blockade of at least one other innate signaling or cytokine pathway at higher doses. IL-1 or RNA-induced signaling are promising targets for intervention.

139 Identification of Dose-Dependent Immune Landscape Signatures Following Administration of Ixo-vec in Non-Human Primates

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Wet age-related macular degeneration (wAMD) is a retinal disease mediated by the abnormal production of vascular endothelial growth factor (VEGF), where the growth of abnormal, leaky blood vessels in the macula leads to a reduction in central vision. The anti-VEGF drug aflibercept can reverse the leakage of fluid and dry the retina but requires frequent bolus injections into the vitreous, which represent a substantial treatment burden to patients and the health care system. Also, the need for frequent bolus injections results in fluctuations in macular fluid that has shown to be associated with long term vision loss. Ixo-vec is a recombinant adeno-associated virus (rAAV) that harnesses ocular cells to become biofactories that express and secrete aflibercept. Ixo-vec demonstrated longer-term aflibercept levels out at least 3 years in the phase 1 OPTIC trial patients. Dose dependent mild to moderate inflammation that was responsive to topical corticosteroid therapy was seen at 2E11 and 6E11 vg/eye doses in the OPTIC study. Phase 2 LUNA study is evaluating 2E11 vg/eye, as well as a new, lower 6E10 vg/eye dose, to expand the known therapeutic window. Findings from nonhuman primate (NHP) studies can help optimize the risk benefit profile in humans. NHP data has demonstrated intravitreal (IVT) administration of Ixo-vec resulted in a non-dose proportional aflibercept levels across several orders of magnitude of Ixo-vec dosing. Levels at 3E10 vg/eye (human equivalent dose [HED] 6E10 vg/eye) were nearly equivalent to aflibercept levels observed at higher doses (up to 2E13 vg/eye). NHP data has also demonstrated dose-dependent inflammation, with little to no inflammation at the 3E10 and 1E11 vg/eye doses to inflammation requiring corticosteroids at 2E12 vg/eye (HED 4E12 vg/eye) or greater. We assessed potential signatures of inflammation including toxicity related to overproduction of exogenous protein and general pathways previously identified in gene therapy studies. Mechanisms were tested by systems-based transcriptomic analysis. Three months following IVT administration of Ixo-vec the choroid, retina, and anterior region tissues of NHPs were isolated for bulk RNA-seq. After determining log-fold changes in gene expression, pathway analysis was performed using three different software tools. The outcomes of these different analyses were highly convergent in identifying the same pathways of interest as dysregulated in a dose-dependent manner. Unfolded protein and endoplasmic reticulum stress responses were not upregulated, indicating that toxicity due to overproduction of exogenous aflibercept did not cause inflammation at higher doses. Furthermore, there was no evidence of disrupted ciliary body architecture or VEGF axis dysregulation. Several immunological pathways previously associated with gene therapy response were upregulated, including engagement of adaptive immune response, already known to develop from ocular and systemic rAAV vector dosing. The inflammatory signatures were dose-dependent with most pronounced upregulation in the anterior region at high doses, followed by the retina, and mostly absent in the choroid. Histological analysis demonstrated dose-dependent increase in mononuclear

infiltrates, including $CD4^+$ T-cells and $CD20^+$ B-cells. In summary, IVT administration of Ixo-vec was well tolerated up to 4E11 vg/eye (HED 8E11 vg/eye) supporting the current dose selections for human wAMD patients in the LUNA trial. These data support the lower doses being explored as well as combination of immunosuppressive prophylactics to suppress formation of adaptive immunity and prevent the development of chronic inflammation for ocular gene therapy.

140 Humoral Arms of the Innate and Adaptive Immune Systems Define the Severity of the Cytokine Storm Response to Human Adenovirus Type 5 Vectors

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The occurrence of severe cytokine storm syndrome constitutes a significant clinical complication that has been observed in a subset of patients post intravenous administration of viral gene therapy vectors. The poor understanding of the molecular mechanisms underlying the development of cytokine storm and the unpredictable variability in the severity of inflammatory responses to an equivalent dose of therapeutic vectors among patients significantly hinders the advancement of new, life-saving therapies. While it is customary to exclude patients with pre-existing neutralizing immunity to a therapeutic vector from clinical trials, this method fails to address the inconsistent and unpredictable nature of the cytokine storm response with regards to its severity and magnitude. We hypothesized that pre-existing non-neutralizing cryptic opsonizing antibodies (CON-Abs), which may arise from past exposure of the patient to phylogenetically distant virus variants and the complement system, may influence the activation of innate phagocytic cells and the subsequent release of inflammatory cytokines following intravenous administration of therapeutic vectors. To experimentally test this hypothesis, we immunized individual groups of wild-type mice with human adenovirus serotypes from species B (HAdv-B3, HAdv-B11), C (HAdv-C2, HAdv-C6), and D (HAdv-D28), followed by intravenous administration of HAdv-C5-based adenovirus vectors. While the antibodies generated in these mice lacked direct neutralizing activity towards HAdv-C5, the levels of inflammatory cytokines released into the bloodstream 6 hours post HAdv-C5 vector administration showed marked variations among mouse groups pre-immunized with different virus serotypes. We further found that CON-Abs, generated towards phylogenetically distinct adenoviruses, were capable of binding to and activating C3 complement fixation on HAdv-C5 viral particles. While immunization of both wild-type (WT) and complement C3 knockout (C3-KO) mice with HAdv-C5 resulted in the generation of virus-specific neutralizing immunity, the severity of the cytokine storm following a subsequent intravenous administration of the HAdv-C5 vector was significantly greater in C3-KO mice compared to WT mice. This highlights that in the in vivo settings, complement C3 serves a suppressive role in the inflammatory response, likely by redirecting adenovirus-immune complexes away from immune phagocytic cells to non-responsive cellular compartments. The results of our study underscore the unexpected impact of CON-Abs on the severity of the cytokine storm response and emphasize the need of devising new methods for pre-screening patients for pre-existing humoral factors that have the potential to enhance inflammatory cytokine responses to therapeutic vectors.Conflict of interest: DSH is a cofounder and shareholder of AdCure Bio, which develops adenovirus technologies for therapeutic use.

141 Causal Role for Immune Response in AAV-Mediated DRG Toxicity in NHPs

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Adeno-associated virus (AAV)-mediated toxicities pose critical safety concerns for patients and are a major obstacle for the gene therapy field. For central nervous system gene therapy, one major toxicity observed is findings of dorsal root ganglion (DRG) degeneration in preclinical models following intra-cerebrospinal fluid (CSF) or high dose intravenous administration. DRG toxicity was one of five AAV-associated toxicities discussed during the 2021 FDA advisory committee meeting. A lesion time course study was conducted to identify molecular events occurring prior to and concurrent with lesion development. An AAV9 variant encoding a human transgene protein was administered to cynomolgus macaques at 3.68E13vg/dose via intra-cisterna magna. Necropsy was performed on Days 5, 9, 19, and 29, respectively. Histopathology confirmed the presence of DRG lesions, including DRG neuronal degeneration/necrosis, DRG mononuclear cell infiltration and nerve fiber degeneration in the spinal cord, starting on day 15 post-AAV administration and fully developing on day 29. However, immunohistochemistry and in situ hybridization revealed the presence of immune cell foci prior to the formation of DRG lesion, as early as day 5 (CD68+ macrophages, NKp46+ natural killer cells, CD20+ B cells, CD4+ T cells) or day 9 (CD8+ T cells), which increased in severity over time on days 15 and 29. Immune cell infiltration prior to neuronal degeneration suggests a causal role of an immune response in DRG toxicity. To test this hypothesis, an immunosuppressant regimen was administered orally in conjunction with intra-CSF AAV delivery. Immunosuppressants reduced the incidence and severity of DRG lesions in NHPs in three independent studies encoding three distinct transgenes. These studies confirm an instrumental role of the immune response in causing DRG toxicity and implicate a specific role for T cells in targeting DRG neurons for cell death. Underlying mechanism(s) for neuronal targeting by immune cells is currently unknown. However, preliminary data suggests transgene overexpression and innate immune pathway activation contribute to neuronal death via cell autonomous and non-autonomous mechanisms.

142 A Human PBMC Assay of Type 1 Interferon Responses to Closely Related AAV Vectors

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Toll like receptor 9 (TLR9) innate immune receptor-mediated responses to AAV gene therapy are emerging as a critical concern. Unmethylated CpG dinucleotides, abundant in unmodified AAV vector genomes, are TLR9 ligands, and are inversely correlated with clinical success. TLR9 signaling leads to type 1 interferon secretion and subsequent Cytotoxic T Lymphocyte (CTL) activation and may contribute to severe adverse events including complement activation associated thrombotic microangiopathy. Unfortunately, many current methods to measure TLR9 responses rely upon limited rodent or cell line models, cannot discriminate between related vectors, and lack an effector cell readout. Here, we present a human PBMC model capable of differentiating between closely related AAV vectors based on the magnitude of the type 1 interferon responses they elicit, and subsequent T cell activation. HEK reporter cells are widely used to predict TLR9 responses to candidate AAV gene therapy vectors. While this model can sufficiently measure TLR9 activation by oligonucleotides, it is poorly stimulated by tested AAV vector doses up to 10^4 MOI. The secretion of type 1 interferons by murine PBMCs is minimal, and vectors are indistinguishable from each other in preliminary experiments. Furthermore, AAV vectors can elicit statistically significant immune signals in human PBMCs, but this is highly donor dependent, and differences are difficult to measure between related vectors. We hypothesized that post AAV gene therapy serum will opsonize candidate vectors, increasing Fc receptor (FcR)-mediated uptake and type 1 IFN signaling. We obtained serum from patients before and after AAV9 gene therapy and observed dose-dependent increases in post therapy serum-mediated neutralization of AAV2 transduction in cell culture, compared to pre-gene therapy serum. We then exposed PBMCs from three healthy donors to empty AAV capsids or AAV2-CAG>GFP. We observed a 19.1pg increase in INF1a secretion after 24 hours in PBMCs exposed to the GFP vector, compared to empty capsid. However, when we first incubated vectors with post gene therapy serum, we observed a >3000% increase in signal, with 727.27pg secreted. Opsonization did not significantly influence empty capsid signal. We then interrogated the serum-mediated effect for FcR-dependence and found that when PBMCs were pretreated with FcR blocking antibodies, the post gene therapy serum-mediated signal increase was blunted. Interestingly, flow cytometry analysis of human PBMCs exposed to post-gene therapy serum-incubated vectors after 72hrs showed increased expression of T-cell activation markers consistent with CTL activation. As post AAV gene therapy seropositive human serum is predicted to be variable, we next attempted opsonization of AAV2 empty capsids, AAV2-CMV>null, and AAV2-CAG>GFP with AAV monoclonal antibodies. We were pleasantly surprised to observe opsonized vector-induced INF1a responses from PBMC donors that were otherwise unresponsive to stimulation, even by oligonucleotide positive control. Moreover, INF1a responses, otherwise indistinguishable by ELISA, correspondingly increased with exposure to unmethylated genomic CpGs ($p<0.0001$) when vectors were first opsonized, and this effect was recapitulated in Luminex readouts for INFg, IL1Ra, IL6, IL8, CXCL10, MCP2 and MCP3. Opsonization of AAV vectors prior to exposure on human PBMCs enables, for the first time to our knowledge, a human-based assay of TLR9-downstream cytokines that can distinguish between vectors that vary by genomic CpG content and could be an important tool for developing vectors that evade TLR9 recognition.

143 Pre-Existing Maternal Immunity to Adeno-Associated Virus Causes Fetal Mortality After In Utero Gene Editing Mediated by Maternal Interleukin 6 and CXCL1

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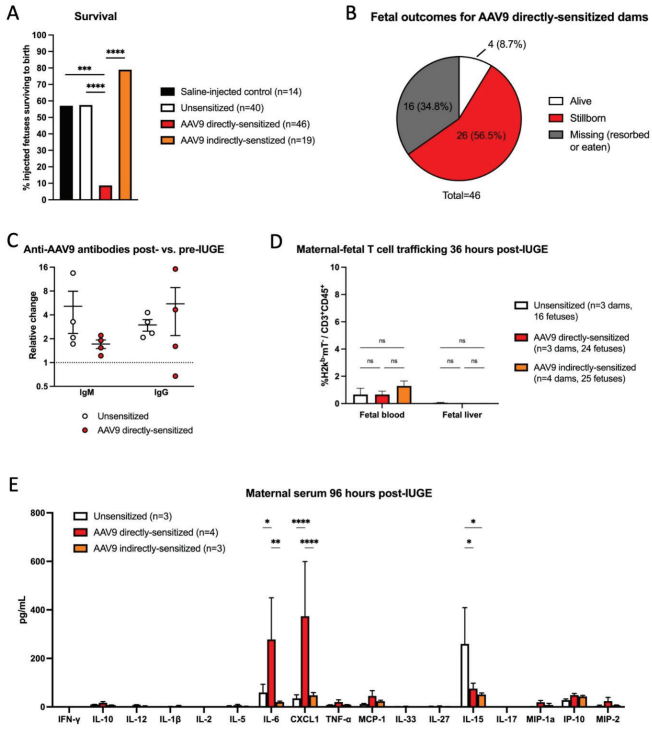
PURPOSE: In utero gene editing (IUGE) is an experimental treatment for inherited metabolic liver diseases. We have previously demonstrated that pre-existing maternal antibodies to AAV impair fetal gene editing in a mouse model of in utero CRISPR-NHEJ (Abstract 2022-A-896-ASGCT). We now investigate the effect of pre-existing maternal immunity on fetal mortality after IUGE and its potential underlying mechanisms.
METHODS: The *ROSA26*^{mTmG} mouse harbors a constitutively-expressed red fluorescent protein (mT) flanked by loxP sites. Successful cleavage at the loxP sites with NHEJ repair and gene deletion results in constitutive expression of green fluorescent protein (mG). We designed an AAV serotype 9 (AAV9) to deliver SpCas9 and a guide-RNA to target the loxP sites (AAV9.SpCas9.sgloxP). Maternal immunity to AAV was evaluated in 3 groups: unsensitized dams (no immunity); dams directly-sensitized to AAV9 by intramuscular injection of AAV9.CMV.null (resulting in active humoral and cellular immunity), and dams indirectly-sensitized to AAV9 by adoptive transfer of serum from directly-sensitized dams (resulting in passive humoral immunity only). IUGE was performed at 16 days post-coitum via fetal intravascular injection of AAV9.SpCas9.sgloxP. Fetal survival to birth was compared to saline-injected controls and among the groups using Fisher's exact test. Maternal immunoglobulin levels pre- and 96 hours post-IUGE were assessed by ELISA. Maternal T cell frequency in the maternal peripheral blood (PB) and draining lymph nodes (LNs) and maternal T cell trafficking into the fetal blood and fetal liver were assessed 36 hours post-IUGE by flow cytometry. Maternal and fetal serum cytokines were assessed 36 and 96 hours post-IUGE using V-PLEX Plus Mouse Cytokine 19-Plex Kit (MSD) and compared using ANOVA.
RESULTS: Maternal direct sensitization to AAV9 significantly increased fetal mortality after IUGE compared to unsensitized dams, indirectly-sensitized dams, and saline-injected controls (Figure 1A), with only 4 of 46 (8.7%) of injected fetuses surviving to birth and stillbirth being the most common type of demise (Figure 1B). Maternal serum anti-AAV9 IgM and IgG levels increased post-IUGE, consistent with exposure and, in the case of directly-sensitized dams, re-exposure of the maternal immune system to AAV9 as a result of fetal injection (Figure 1C). No differences in maternal T cell frequency, including activated $CD4^+$ or $CD8^+$ T cells, in the maternal PB or draining LNs were observed among the groups. Additionally, pre-existing maternal immunity to AAV9 did not result in increased trafficking of maternal T cells into the fetal blood or liver following IUGE (Figure 1D). However, directly-sensitized dams had significantly elevated levels

of 2 pro-inflammatory cytokines/chemokines, IL-6 and CXCL1 (both of which are associated with stillbirth/preterm labor) (Figure 1E). Elevated levels of IL-6 were similarly present in their fetuses. **CONCLUSION:** Pre-existing maternal immunity to AAV significantly increases fetal mortality after IUGE. The mechanism is re-exposure of the primed maternal immune system to AAV resulting in an inflammatory response characterized by interleukins/cytokines implicated in the pathogenesis of stillbirth. Pre-procedural evaluation of pre-existing maternal AAV immunity will be critical for ensuring the safe application of IUGE with AAV vectors to the clinical setting.



846 Reducing Cas9 Immunogenicity *In Vivo* Leveraging Cell-Endogenous Protein Degradation Pathway

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There is growing interest in the therapeutic use of CRISPR-Cas9 to correct monogenic human disorders, with a number of medicines progressing rapidly into clinical trials. However, "the golden standard" Cas9 isolated from Streptococcus pyogenes (SpCas9), is known to trigger host immunogenicity. It is reported that the continuous cytosolic presence of exogenous proteins can increase antigen presentation via MHC-I. This represents the foundation of cellular immunity, leading to the activation of cytotoxic T-Cell response. Evading cellular immunosurveillance could represent a major advancement to broaden the clinical applications of CRISPR. Here, we rationally engineered SpCas9 with a single amino acid substitution in the Rec2 domain to introduce a chaperone-mediated autophagy (CMA) binding moiety. This pentapeptide domain is recognized by the HSC70 chaperone that binds and directs target proteins to lysosomal degradation. We reasoned that a forced and selective lysosomal degradation would make SpCas9 less detectable by the MHC-I, thus reducing the antigen presentation and the subsequent CD8+ T-cell activation. We prove that the mutated SpCas9 presented an extremely low intracellular concentration after transfection in primary and immortalized cells, a feature specifically due to an acquired high degradation rate. We, therefore, called this novel mutant Fast Degrading Cas9, or simply FaDe Cas9 (fdCas9). In vitro kinetics of SpCas9 and fdCas9 were undistinguishable, indicating that the amino acid substitution does not affect the enzymatic structure or function. Moreover, the in vivo tail vein Adenovirus-delivered fdCas9 (AdV-fdCas9) demonstrated comparable editing levels with SpCas9, thus making fdCas9 the first SpCas9 mutant successfully used for somatic genome editing in adult mice. Most importantly, mice pre-exposed to fdCas9 and then infected with AdV-fdCas9 showed a markedly reduced activation of cellular immunity as measured by cytotoxic CD8+ T-cell infiltrates in the liver when compared to mice pre-exposed to SpCas9. Similarly, human peripheral blood monocyte cells (PBMCs) showed a reduced T-cell response when challenged in an ELISpot with fdCas9 compared to SpCas9. Taken together, we generated a novel version of SpCas9 that circumvents activation of host immunity, making it an attractive enzyme for therapies that require in vivo administration of CRISPR agents to patients.

1006 Immune Responses Observed to AAV Gene Therapy for the Treatment of GM2 Gangliosides

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Tay-Sachs Disease and Sandhoff disease are neurodegenerative diseases caused by lysosomal storage of GM2 gangliosides. Previously in an expanded access trial, a two vector rAAVrh8 gene therapy (rAAVrh8 HexA/HexB) was tested and found to be safe. The current trial is additionally highlighted in ASGCT abstract (#2023-A-829-ASGCT). In brief, patients were injected with bilateral intrathalamic delivery (BiTh) and with intracisternal/intrathecal (CSF) injection at three different dose ranges: starting BiTh dose (STD) of 180 mcl (5.87E+12vg BiTh; 1.42E+14vg total), a low-dose (LD) of 360 mcl (1.17+E13vg BiTh; 1.95E+14vg total), and a mid-dose (MD) of 720 mcl (2.35E+13vg BiTh; 2.18E+14 total). Patients were treated with an immunosuppressive regimen that included rituximab, corticosteroids (3 months post-delivery) and sirolimus (6 months post-delivery). Despite lack of measurable circulating CD19 and CD20 positive cells all 6 patients treated thus far seroconverted to neutralizing antibody (Nab) titers of at least 1:100 by 30 days post-delivery with some Nab titers as high as 1:1000. Nab titers were fairly stable between 30- and 180-days post-therapy. In addition to positive B-cell responses, capsid specific T-cell response were measured by interferon-gamma ELISPOT assay. All 6 patients had a positive response to AAV capsid post-injection, and all but one patient had a positive response between day 15 and day 28 post-injection. Interestingly, the patient 1 (STD) with the lowest dose did not have a positive response to AAV capsid by ELISPOT assay

until day 83 post-injection. Patient 4 (LD) had significant elevations of liver transaminases, AST and ALT, concurrently with positive IFNγ ELIPSOTs around day 14 post-injection. The patient was treated with solumedrol at 10mg/kg/day for three days and resulting T-cell responses and transaminases resolved. Additionally, other patients (LD and MD) had less pronounced elevations of both transaminases and ELISPOT responses and were treated with increases of prednisolone dosage from 1mg to 2mg/kg, resulting in reduction of IFNγ spot forming units. No positive responses were observed by ELISPOT with peptide pools generated from the transgene. Further flow cytometry characterization was run on PBMCs either directly or after culturing with AAV capsid peptides pools. Several patients at different time points showed expansion of the Treg populations after culturing with AAV capsid peptide pools suggesting induction of AAV capsid specific Tregs. To further understand local immune responses, cytokines were measured in the CSF of patients, at pre-treatment as well as 3- and 6-months post AAV infection. In all patients, CXCL10 was elevated from pre-treatment levels at 3-months and even further elevated at 6-months post-injection suggestive of a local IFN-gamma response. None of the other cytokines measured including IFNγ, IL-4, MCP-1, IL-8, IL-2, IL-1β, TNFα, IL-17A, IL12p70, TGFβ, IL-10 or IL-6 or were significantly changed. Taken together, despite the delivery route and substantial immunosuppression regimen, notable immune responses, including T and B-cell responses to AAV capsid, were observed in all patients after CNS targeted AAV gene therapy.

Next Generation CAR, TCR, and AAV Technologies for Solid Tumors

144 Adeno-Associated Virus-Mediated Targeting of HER2+ Brain Metastasis

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Twenty percent of breast cancers overexpress human epidermal growth factor receptor 2 (HER2), an oncogenic protein, and up to 50% of patients with HER2+ breast cancer develop central nervous system (CNS) metastases. The median survival of patients with HER2+ breast cancer brain metastasis (BCBM) undergoing standard-of-care treatment is 10-25 months. Trastuzumab (Herceptin®), a humanized anti-HER2 antibody, is part of the standard-of-care adjuvant regimen for patients with HER2+ disease. However, the efficacy of intravenously (IV) infused trastuzumab against intracranial metastases is limited since it does not fully cross the (intact) blood-brain barrier. Intrathecal delivery of trastuzumab improves the prognosis of patients with HER2+ BCBM, but chronic management of the disease through this approach is challenging due to the required frequency and complexity of the CNS dosing regimen. To address these shortcomings, we developed adeno-associated virus (AAV) vectors carrying codon-optimized versions of trastuzumab designed to achieve effective and stable drug concentrations in the brain following a single CNS delivery. We evaluated the anti-tumor efficacy of AAV-trastuzumab vectors in several orthotopic cell line xenograft mouse models of HER2+ BCBM (including BT-474 cell lines sensitive or resistant to trastuzumab and the MDA-MB-453 cell line). Compared with *Rag1* knockout (KO) mice treated with an AAV-isotype control vector, single intracerebroventricular (ICV) administration of AAV-trastuzumab at 10^{11} genome copies/mouse significantly extended survival in both BT-474 cell line xenograft models ($p < 0.0001$). The MDA-MB-453 xenograft model animals exhibited high sensitivity to AAV-trastuzumab treatment, including complete remission of brain xenografts in 100% of the animals. Dose-titration studies comparing ICV with IV injection of AAV-trastuzumab vector in healthy *Rag1* KO mice highlighted the benefit of direct administration to the CNS as ICV-administered mice had a ~4-fold increase in trastuzumab protein expression (by ELISA) in the brain compared with IV-administered mice receiving the same dose (10^{11} GC/mouse) with mass spectrometry analysis confirming this trend. Western blot analysis of brain lysates from AAV-trastuzumab-treated *Rag1* KO mice suggested that heavy and light IgG_1 chains were expressed at equimolar concentrations. These preclinical findings highlight the potential of an ICM delivered, AAV-based gene therapy to effectively deliver trastuzumab to the CNS for the treatment of HER2+ BCBM.

145 Safety Evaluation of Intra-Cisterna Magna (ICM) Delivery of a Novel AAV-Trastuzumab Vector to Target HER2+ Breast-to-Brain Metastasis in Rhesus Macaques

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In 2020, breast cancer surpassed lung cancer and became the most diagnosed malignancy worldwide. In the United States, breast cancer is the second leading cause of cancer-related deaths in women (43,500 in 2022). About 15-20% of breast tumors overexpress human epidermal growth factor receptor 2 (HER2), an oncogene and effective therapeutic target for the treatment of breast cancer. HER2-targeting using monoclonal antibodies such as trastuzumab (Herceptin®) has revolutionized breast cancer treatment and improved overall survival by controlling systemic disease. However, up to 50% of patients with advanced HER2+ breast cancer develop central nervous system (CNS) metastases, with an overall survival of 26 months. An intact blood-brain barrier limits the efficacy of systemic HER-2-targeted antibodies in the CNS by preventing large molecule diffusion into the brain parenchyma. To overcome this limitation, we developed a novel codon-optimized adeno-associated virus (AAV) encoding trastuzumab to treat HER2+ CNS disease. Intracerebroventricular delivery of AAV-trastuzumab vectors has demonstrated therapeutic efficacy against orthotopic xenograft mouse models of HER2+ breast cancer-to-brain metastasis (BCBM). To further evaluate the safety and transgene expression of AAV-trastuzumab vectors, we administered intra-cisterna magna (ICM) injections containing either the CB7 promoter (a synthetic promoter derived from chicken b-actin promoters with a CMV enhancer element) or UbC promoter (constitutive human ubiquitin C promoter) in adult female Indian rhesus macaques with pre-existing neutralizing antibodies against AAV (n=2/vector; $3x10^{13}$ genome copies/animal). These promoters can drive sustained expression of transgenic proteins in non-human primate brain regions; therefore, we compared CB7 vs. UbC promoter

strength in AAV-trastuzumab vectors in this model. Animals were euthanized at day 35 post-treatment and jugular perfusion of the brain with saline was performed. No clinical signs of toxicity were observed during the study. Biodistribution analyses showed 10- to 1000-fold-higher levels of vector genome copies and transgene RNA transcripts in the brain compared with the heart and liver. Presence of trastuzumab transcripts in the CNS was confirmed by *in situ* hybridization and single-nuclei RNA sequencing (0.6%-1% of cells in the cerebellum were positive). We quantified trastuzumab protein levels in the tissue lysates, cerebrospinal fluid (CSF), and serum of treated monkeys by ELISA, which detected the presence of trastuzumab in multiple brain regions (1-6 ng trastuzumab/mg protein) and the spinal cord (up to 40 ng trastuzumab/mg protein). Trastuzumab levels in the CSF reached 8-64 ng/mL at day 35 in macaques injected with vector containing the UbC promoter. However, in animals injected with transgene driven by the CB7 promoter, CSF trastuzumab levels peaked at 16-32 ng/mL between days 14 - 21 post-injection but were undetectable by the end of the study. Weak or no signal above background was observed for serum levels of trastuzumab, regardless of the vector administered. Virtually no anti-drug antibody (ADA) responses against trastuzumab were detected in the CSF by ELISA, although 3/4 monkeys displayed levels of 1.6-13.6 μg/mL of ADA in the serum prior to euthanasia. Overall, ICM delivery of AAV-trastuzumab vectors driven by the UbC promoter was well tolerated and led to transgene expression in the CSF and brain tissues of rhesus macaques. Importantly, CNS trastuzumab levels within the range of what was observed in macaques show significant antitumor activity against orthotopic cell line-derived xenograft mouse models of BCBM. Based on these favorable preclinical results, we believe this treatment approach for patients with advanced HER2+ BCBM could be advanced to clinical trials.

146 Designed Tumor Microenvironment Responsive Biosensors Enhance Chimeric Antigen Receptor T Cell Therapy for Cancer

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Chimeric antigen receptor (CAR) T cell therapies produce long-lasting responses in a subset of patients with B cell malignancies. However, success is limited in cancers with complex tumor microenvironments (TMEs) that impact CAR T cell persistence and function. Here we hypothesized that co-engineering CAR T cells with a second chimeric receptor (biosensor) that converts TME-associated input into signaling output that mediates enhanced CAR T cell expansion, memory and long-term persistence can improve outcomes of CAR T cell therapy. We developed a novel computational-experimental approach for the *de novo* bottom-up assembly and design of biosensors with programmable input-output behaviors and their full functional assessment in vitro and in vivo. We designed biosensors responding to vascular endothelial growth factor (VEGF) or colony stimulating factor 1 (CSF1) because of their key roles, elevated levels and association with poor prognosis in several cancers. As orthogonal signaling endodomain, we leveraged parts of the thrombopoietin receptor c-MPL which simultaneously produces co-stimulatory and γ-chain cytokine signals upon activation. The computational assembly of ligand binding domains (derived from VEGFR2 or CSF1R), linker, transmembrane and endo-domains (derived from c-MPL) generated a set of biosensor variants with programmable strength (low vs high intensity) and behavior (ligand-dependent vs baseline constitutive activity) for experimental evaluation. Biosensors targeting VEGF (VMR) or CSF1 (CMR) were highly expressed in activated human T cells upon retroviral transduction. Signaling potency of VMR and CMR variants was determined by measuring phosphorylated STAT5 levels in response to ligand (VEGF or CSF1) and correlated with the computationally predicted signal transduction propensity. Activation of VMR and CMR both mediated T cell persistence in long-term T cell cultures mimicking the effect of homeostatic γ-chain cytokine signals. To determine the impact of VMR and CMR on T cell function in response to target tumor cells we co-expressed our biosensors with CARs targeting Ephrin type A receptor 2 (EphA2) or B cell maturation antigen (BCMA) respectively. When challenged with the lung cancer cell line A549 or the multiple myeloma cell line MM.1S in vitro, VMR+ and CMR+ CAR T cells expanded and persisted significantly better in the presence of VEGF or CSF1 compared to CAR T cells without biosensor. In vivo, treatment with VMR+ CAR T cells significantly controlled VEGF high A549 metastatic tumors in mice and enhanced their overall survival compared to CAR T cells without VMR ($p<0.0001$, n=10 mice/ group). Similarly, CMR+ CAR T cells were more potent at tumor control than CAR T cells without CMR in a MM.1S-based xenograft mouse model of MM with high CSF1 expression ($p<0.0001$, n=10 mice/ group). In conclusion, we developed and validated a novel bottom-up computational-experimental approach for the assembly and design of multi-domain protein biosensors with programmable input-output behavior. We demonstrate that our strategy can overcome major challenges posed by the TME and results in potent and tumor selective enhancement of CAR T cell therapy for cancer.

147 Development of Adeno-Associated Virus (AAV) Vectors for Intratumoral Gene Replacement Therapy in a Novel Mouse Model of Neurofibromatosis Type 1 (NF1)

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Introduction: Neurofibromatosis type 1 (NF1) is one of the most common human monogenetic diseases, with an incidence of 1 in 3000. The NF1 tumor suppressor gene encodes for the GTPase-activating protein, neurofibromin. Biallelic loss of *NF1* causes uninhibited GTPase RAS activity resulting in tumor proliferation. The current standard of care for these tumors, which include neurofibromas, gliomas and malignant peripheral nerve sheath tumors (MPNSTs), is limited to surgical debulking and small molecule inhibitors which are not curative. Development of gene replacement therapy for this disease has been hindered by the large NF1 coding sequence (~8.5

kb) which exceeds the packaging capacity of AAV vectors. Secondly, neurofibromas are diffusely distributed and arise from Schwann cells in an avascular endoneurium, making access of AAV to these cells challenging. **Methods**: Here, we describe a gene replacement approach utilizing two novel AAV9-NF1 platforms. One platform is a dual trans-splicing AAV9 system comprised of vectors that split the human NF1 cDNA to accommodate its size. Specifically, one vector contains the promoter upstream and a splice donor downstream of the 5' NF1 cDNA. The second vector contains the 3' NF1 cDNA utilizing an upstream splice acceptor site and a downstream HA tag. These vectors concatemerize into full length NF1 mRNA. Our other strategy utilizes an AAV encoding a mini-NF1 transgene (AAV-NF1^{mini}) which is the neurofibromin GTPase activating protein domain. Since the expected product will be a truncated form of neurofibromin, we added a HA-tag for *in vivo* detection. Vector transduction efficiency was measured by infecting MPNST cell lines for 72 hours and analyzing expression of full length NF1 protein and HA tag against a GFP-vector control on western blot. Tumor suppressive functionality of AAV-NF1 constructs in MPNST cells was assessed using cell proliferation and viability assessments. To evaluate our vectors *in vivo*, we created an induced neurofibroma model in the mouse sciatic nerve by injecting AAV1-Cre to *Nf1*$^{R681*/4F}$ mice carrying an inactive (*Nf1*^{R681*}) and a floxed (*Nf1*^{4F}) allele. We evaluated tumor growth at 5 and 12 weeks with 7T MRI and performed histological and immunohistochemical tumor analysis using S100*B* and Ki67 staining.**Results**: Both AAV-NF1^{dual} and AAV-NF1^{mini} showed detectable NF1 protein and HA tag, respectively, on western blot. Both systems are functional in transduced MPNST cell lines by decreasing the phospho-ERK1/2/total ERK1/2 ratio, (i.e. reducing RAS pathway activity). Additionally, both systems significantly inhibit cellular viability and proliferation of MPNST cell lines. Our Cre-inducible neurofibroma tumor model showed evidence of increased sciatic nerve volume in the right sciatic nerve when compared to non-injected contralateral nerve on 7T MRI. Histological evaluation of the AAV1-Cre injected sciatic nerve revealed spindle cells with increased cellularity and with S100*B* and Ki67 positivity, indicating tumor formation. **Conclusion**: Our data revealed that both AAV-NF1 platforms can restore neurofibromin function *in vitro* by reducing RAS pathway activity. Additionally, both systems restore tumor suppressive function in MPNST cell lines. We have established a localized induced NF1 mouse neurofibroma model in the sciatic nerve. This model will be used to test the therapeutic efficacy of intratumoral injection of AAV-NF1 vectors. Our goal is to rapidly translate this approach to treat plexiform neurofibromas that are challenging to treat surgically and/or fail to respond to Selumetinib.

148 Enhanced Anti-AML Potency of DARIC33 by iSynPro-IL-15*: An IL-15 Expression Module Driven by a Tightly Regulated Synthetic Promoter Activated by Antigen Receptor Signaling

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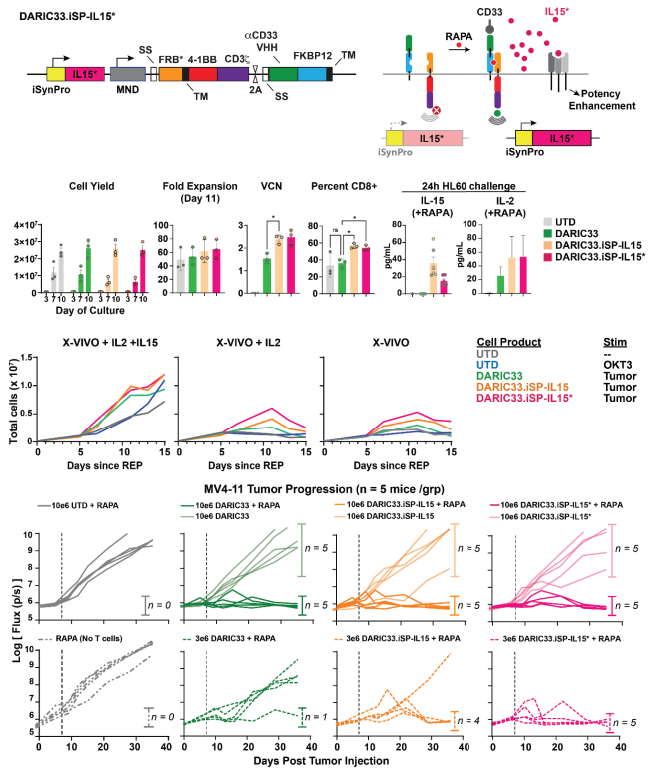
INTRODUCTION: DARIC33, a dimerizing agent regulated immunoreceptor complex targeting CD33 that requires low doses of rapamycin (RAPA) for activity, is being studied in an ongoing phase 1 clinical trial (NCT 05105152). Genetic enhancements of CAR T cell potency may improve anti-leukemic efficacy but risk adverse effects, such as uncontrolled cell growth. We previously found that while intermittent administration of IL-15 to non-human primates is well tolerated and expands memory T cells, continuously delivered high-dose IL-15 is toxic (Berger et al, Blood 114(12):2417). Thus, we hypothesized that combining drug-controlled DARIC33 activation with regulated IL-15 secretion could enhance anti-AML potency without driving autonomous T cell growth or severe toxicity. **RESULTS:** We designed genetic modules in which, following antigen signaling a novel inducible synthetic promoter (iSynPro, iSP) transiently drives transcription of human IL-15 (iSP-IL-15) or IL-15 modified to restrict signaling to cells expressing IL-15Ra (iSP-IL-15*). We then manufactured DARIC33 CAR T cells using lentiviral vectors with or without integrated iSP-IL-15 modules. *In vitro* cell expansion, vector copy number, and CD33 antigen-binding capacity were similar among T cells transduced with DARIC33, DARIC33.iSP-IL-15 or DARIC33.iSP-IL-15*. All cell products contained high proportions of naïve T cells (CD62L+/CD45RO+). Notably, iSP-IL-15 and iSP-IL-15* augmented products exhibited higher CD8+ T cell proportions. Following *in vitro* challenge with AML cells, DARIC33.iSP-IL-15 and DARIC33.iSP-IL-15* exhibited RAPA-dependent secretion of IL-15 and other effector cytokines. Next, we cultured antigen-stimulated DARIC33 cell products in IL-2/IL-15 supplemented media and observed continued T cell expansion until day 21, highlighting the potential of IL-15 to expand T cells. Strikingly, when IL-2 and IL-15 were omitted from the culture media, both iSP-IL-15* and iSP-IL-15 DARIC33 products demonstrated enhanced expansion but normal contraction kinetics. These results illustrate the capability of the iSP transcription element to enhance T cell function through tightly regulated IL-15 production without promoting unrestrained T cell growth. Finally, we treated AML tumor bearing mice with untransduced (UTD) control T cells or graded doses of DARIC33 or DARIC33.iSP-IL-15/IL-15*. While both 10e6 DARIC33 and DARIC33.iSP-IL-15* T cells controlled tumor growth, when the effector cell dose was decreased to 3e6/mouse, both DARIC33.iSP-IL-15 and DARIC33.iSP-IL-15*continued to suppress tumor growth, whereas DARIC33 did not. Day 35 necropsies of DARIC33.iSP-IL-15* treated mice did not show tumors or evidence of uncontrolled T cell proliferation. **CONCLUSION**: We find iSynPro-IL-15* retains properties of inducible

expression and potency enhancement. Our results suggest that the triple safety features of iSynPro-regulated expression, IL-15* safety modification, and RAPA controlled DARIC33 activation prevent unrestrained T cell outgrowth. We believe this potency enhancement strategy will be applicable to next generation clinical AML CAR T cell therapeutics.



149 Preclinical Development of AB-1015, an Integrated Circuit T Cell Therapy Containing an ALPG/MSLN Logic Gate and FAS/PTPN2 shRNA-miR, for the Treatment of Ovarian Cancer

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CAR T cell activity in solid tumors is limited by off-tumor toxicity, antigen heterogeneity, poor persistence, and functional suppression resulting from the tumor microenvironment (TME). To address these challenges, we have developed AB-1015, an autologous, integrated circuit T (ICT) cell product for the potential treatment of ovarian cancer. The AB-1015 transgene cassette includes two functional modules: an "AND" logic gate designed to limit off-tumor toxicity through dual tumor antigen recognition, and a dual shRNA-miR targeting FAS and PTPN2 to resist TME suppression and to improve ICT cell function. The AB-1015 DNA cassette is inserted into the T cell genome at a defined genomic site, GS94, via CRISPR integration of transgenes by electroporation (CITE). The AB-1015 logic gate consists of a priming receptor (PrimeR) against ALPG/P and an inducible MSLN-targeted CAR that is upregulated in response to PrimeR engagement. The dual-antigen specificity of the logic gate was assessed in vivo using a dual flank tumor xenograft model where one tumor expressed both ALPG and MSLN, and the contralateral tumor expressed MSLN alone. Compared with the RNP-treated group, the constitutive, benchmark anti-MSLN CAR demonstrated tumor reduction on both flanks. In contrast, the activity of AB-1015 was specific to the ALPG+MSLN+ tumor. To model priming antigen heterogeneity that AB-1015 may encounter in the tumor, we utilized an admixed co-culture system where varying proportions of ALPG+MSLN+ target cells were spiked into cultures that were otherwise MSLN+. AB-1015 was able to eliminate admixed co-cultures where as few as 5-15% of the target cells expressed ALPG+MSLN+. AB-1015 also contains a dual shRNA-miR that targets FAS and PTPN2, two critical mediators of T cells survival and function. FASL, the cognate ligand for FAS receptor, is expressed on the surface of activated T cells and is significantly overexpressed in the ovarian cancer TME. AB-1015 demonstrated resistance to FAS-mediated apoptosis in vitro. Knockdown of PTPN2, a phosphatase involved in T cell proliferation and functional persistence, resulted in enhanced AB-1015 cell expansion during an in vitro repetitive stimulation assay over a period of 14 days, as well as a 30-fold reduction in tumor outgrowth compared with logic gated T cells alone. Furthermore, the anti-tumor activity of AB-1015 was established in an intraperitoneal OVCAR3 ovarian xenograft model that resembles high-grade serous ovarian cancer histology and dissemination within the peritoneal cavity. AB-1015 demonstrated potent anti-tumor activity as demonstrated by decrease in bioluminescent signal from the tumors treated with AB-1015. To further increase the stringency of our preclinical models, we engineered the subcutaneous MSTO xenograft model to express FASL. In this model, benchmark anti-MSLN TCR Fusion Construct T cells (TRuC-T cells) failed to control tumor outgrowth. In contrast, AB-1015 resists FASL suppression via shRNA knockdown of FAS receptor on the ICT surface. As a result, AB-1015 is capable of completely clearing these otherwise difficult-to-treat tumors in this model. In summary, AB-1015 is specific for ALPG/P+MSLN+, demonstrates superior potency, expansion, and persistence compared with logic gated T cells alone, and is resistant to ovarian TME suppression in preclinical studies. Based on these promising preclinical data, AB-1015 is being studied in a phase I clinical trial (NCT05617755) to assess the safety, pharmacokinetics, immunogenicity, and efficacy for patients with platinum-resistant ovarian cancer.

150 Engineering Pharmacologically Relevant, FDA-Approved Small-Molecule-Regulated Gene Circuits for Therapeutic Applications in the Brain

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Background: First generation engineered cell and gene therapies have had success in the treatment of certain cancers and genetic diseases. However, limitations in their efficacy, safety and control demonstrates a clear need for improvement to expand their therapeutic potential. Small molecule (SM)-regulated gene circuits, such as transcriptional switches, can be used to address these challenges, but often do not function at pharmacologically feasible drug concentrations, a critical limitation preventing their clinical application. In addition, many SM drugs used to control gene circuits are unable to penetrate the blood-brain barrier (BBB), preventing their use in therapeutics for diseases affecting the brain. Development of SM transcriptional switches that are regulated at pharmacologically relevant concentrations of BBB-penetrant SM drugs would enable novel and previously unattainable regulated cell and gene therapies for the brain. **Methods:** We created a Regulator Dial (RegDial) gene circuit responsive to tamoxifen metabolites (endoxifen) by engineering a novel inducible promoter responsive to a synthetic transcription factor (synTF). The inducible promoter comprises zinc-finger binding sites upstream of a minimal promoter. The synTF contains a corresponding zinc-finger DNA binding domain fused to a transcriptional activator and ERT2, a modified estrogen receptor domain that translocates from the cytoplasm to the nucleus upon binding to endoxifen. Steady state concentrations of free endoxifen in the human brain were estimated based on human and rodent PK data resulting in a target sensitivity for our synTF of 2.5 nM endoxifen. The synTF was evaluated for function at a range of endoxifen concentrations in U87MGs with an inducible reporter and showed activity at concentrations > 25 nM, well above the desired 2.5nM threshold. To address this, we computationally predicted mutations based on the ERT2/endoxifen structure that could increase endoxifen binding affinity and screened the mutants for their ability to enhance the SM sensitivity of the synTF. **Results:** We designed and tested 157 ERT2 mutations in the context of the synTF, of which 15 initiated transcription of the reporter at 10 nM endoxifen. To further improve sensitivity, we created a combinatorial library of the 15 lead mutations and sorted by FACS for library members that activated a reporter beginning at 0.1 nM endoxifen. Mutants from the sorted population were identified by long-read NGS and evaluated for their ability to induce expression of a therapeutic payload, IL-12, from their cognate promoter in vitro. The lead mutant induced expression of IL-12 by over 10 fold at 1 nM endoxifen (from baseline of 8,400 pg/mL to 88,400 pg/mL per $1x10^6$ cells in 24h), well below the expected concentration of endoxifen in the brain. We evaluated the in vivo function of the RegDial with our lead ERT2 mutant by implanting NSG mice with engineered cell lines expressing the improved synTF and a nanoluciferase reporter gene. We observed significant induction of nanoluciferase luminescence in endoxifen treated mice compared to the vehicle-only condition. **Conclusions:** We have engineered a novel RegDial gene circuit that has sensitivity to an FDA-approved SM drug at pharmacological concentrations, enabling its function in a wide range of tissues, including the brain. This circuit robustly induces a therapeutic payload, IL-12, and functions in vivo in a mouse model. This technology can be used to potentially enhance the efficacy, safety, and control of a broad range of cell and gene therapies.

Gene Targeting and Gene Correction: Liver

151 Effective Treatment of Mitochondrial Complex III Deficient Mice with Hepatocyte-Targeted Gene Therapy

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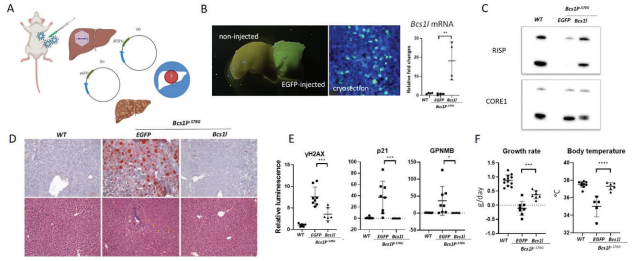
Mitochondrial disorders comprise a large group of monogenic inborn errors of metabolism, typically with compromised respiratory chain (RC) function, leading to a wide spectrum of manifestations from myopathies to encephalopathies and multi-organ disease. Mutations in the RC complex III (CIII) assembly factor gene *Bcs1l* are the most common cause of CIII deficiency. The most severe of them is GRACILE (Growth Restriction, Aminoaciduria, Cholestasis, liver Iron overload, Lactic acidosis, and Early death) syndrome. GRACILE syndrome patient mutation (Ser78Gly) knock-in mice show severe juvenile-onset disease with hepatopathy, kidney tubulopathy, hypoglycemia, growth restriction, kyphosis, and decreased bone mineral density and fat mass. It is unknown to which degree the systemic and metabolic phenotypes such as growth restriction, increased lipolysis and low blood glucose dependent on the liver. To interrogate the contribution of the metabolically compromised hepatocytes, we used recombinant adeno-associated viruses (rAAVs), serotype 9 and a hepatocyte-specific human ApoE enhancer and α1-antitrypsin promoter, to express wild-type BCS1L in the *Bcs1l*$^{p.S78G}$ liver starting at presymptomatic age (P21, Fig A). A single intraperitoneal injection of rAAVs targeted the liver efficiently, as shown by EGFP fluorescence in the whole liver and in frozen sections. qPCR showed approximately 20-fold increased total *Bcs1l* mRNA expression in the rAAV-Bcs1l injected liver (Fig. B). Blue Native Gel Electrophoresis (BNGE) and Western blot analyses of isolated liver mitochondria showed that CIII assembly was restored (Fig C) by rAAV-Bcs1l, as indicated by the ratio of RISP to CORE1 subunit (n=4). H&E and Oil Red O staining showed that liver histopathology and fat accumulation, respectively, were largely prevented (Fig. D). The DNA damage marker phosphorylated histone H2AX (γH2AX) decreased by more than fifty percent, while the upregulation of the cell cycle arrest marker p21 (CDKN1A) and senescence marker GPNMB were completely abolished by rAAV-Bcs1l. Strikingly, the liver-

targeted gene therapy was sufficient to correct systemic phenotypes such as hypothermia and decreased growth, indicating that these manifestations are indeed highly dependent on CIII function in the liver. We propose that liver-targeted gene therapy is an effective strategy to treat mitochondrial disease phenotypes with mainly visceral manifestations, such as GRACILE syndrome.



Effective treatment of mitochondrial complex III deficient mice with hepatocyte-targeted gene therapy A) Hepatocyte targeted gene therapy strategy to introduce wild type *Bcs1l* expression in *Bcs1l*$^{h.S78G}$ mice. As a control, rAAVs expressing *EGFP* was injected. B) The hepatocyte specific *EGFP* expression was confirmed both in the tissue level and in the cryosections. The qPCR data shows the increase in the mRNA level expression of *Bcs1l*, as a result of rAAV- *Bcs1l* injection. C) BNGE data shows a dramatic increase in the availability of RISP per CORE1 (a component of CIII). D) Oil Red O staining (the upper panel) on the liver histological sections showed that the fat accumulation was completely prevented and H&E staining (the lower panel) shows the absence of progenitor cells (dashed boundary) , apoptotic cells (green arrow), the empty spaces created by fat accumulation (yellow arrow) after rAAV- *Bcs1l* injection. E) The level of γH2AX, p21 and GPNMB, which are the markers of DNA damage, cell cycle arrest and senescence, respectively, was partially of fully rectified by the gene therapy. F) The systemic phenotypes like, growth rate and body temperature were corrected by the liver targeted gene therapy.

152 An Epigenetic Hit-and-Run Platform for Durable and Effective Silencing of Pcsk9 *In Vivo*

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Epigenome editing is emerging as a promising approach for DNA break-free and durable gene silencing. In this regard, we have previously developed Engineered Transcriptional Repressors (ETRs), chimeric proteins containing a programmable DNA Binding Domain (DBD) fused to either one of the following epigenetic Effector Domains (EDs): KRAB, the catalytic domain of DNMT3A and DNMT3L. Once transiently delivered, this triple ETR combination can induce long-lasting gene repression, both in cell lines and primary cells, through *de novo* deposition of targeted DNA methylation. Here, we challenged the ETR technology to *in vivo* application, focusing on *Pcsk9*, a gene involved in cholesterol homeostasis and whose hepatic inactivation is under investigation for the treatment of hypercholesterolemia. We initially compared efficacy of different ETR architectures containing either dCas9, TALE or ZFP DBDs in a *Pcsk9*-reporter cell line. These studies identified a ZFP-based ETR combination capable of inducing robust (>90%) and durable (up to 50 days) epi-silencing of *Pcsk9*. We then assessed the specificity profile of the selected ZFP-ETRs by combining whole-transcriptome and genome-wide DNA methylation analyses. Here, we found that ETR-treatment resulted in minimal transcriptional and epigenetic perturbations besides *Pcsk9*. These findings are in line with those reported in recent studies for similar epi-editing platforms. Motivated by these data, we packaged the ZFP-ETRs into a pre-selected lipid nanoparticle (LNP) and administered it to adult mice. Longitudinal analysis showed a rapid reduction in circulating levels of Pcsk9 which stabilized at ~50% of vehicle-treated levels for up to 1 year. These reductions were accompanied by *de novo* methylation at the *Pcsk9* promoter. To extend these findings, we subjected a second cohort of *Pcsk9*-silenced mice to Partial Hepatectomy (PH), a surgical procedure that promotes liver regeneration through hepatocyte proliferation. Notably, epi-silencing of *Pcsk9* was resilient to PH. In line with this data, methylation profile of the *Pcsk9* promoter from liver biopsies of mice before and after PH showed comparable levels of *de novo* DNA methylation. Finally, with the aim of reducing the molecular complexity of the triple ETR combination, we built and tested in the *Pcsk9*-reporter cell line dozens of new ETR architectures based on a single ZFP DBD, identifying new ones with improved on-target and no off-targeting activity over the standard triple ETR combination. We are profiling specificity of the remaining ETRs *in vitro* and testing *in vivo* their epi-silencing activity. In conclusion, we provided here proof-of-principle of efficient and durable epi-silencing of Pcsk9 *in vivo* upon transient delivery of ETRs, laying the foundation for the development of novel gene silencing approaches.

153 Optimizing Therapeutic Base Editing for Hereditary Tyrosinemia Type 1

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Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive metabolic liver disease that can cause death in the first months of life and incurs an increased risk of hepatocellular carcinoma. It results from mutations in the *FAH* gene which encodes fumarylacetoacetate hydrolase, the last enzyme in the tyrosine catabolic pathway, resulting in accumulation of toxic metabolites. Current treatment for HT1 involves strict adherence to daily NTBC, a repurposed herbicide that inhibits 4-hydroxyphenylpyruvate dioxygenase (HPD), the second enzyme in the tyrosine catabolic pathway, and dietary restriction of tyrosine and phenylalanine. Noncompliance rates are high, and liver transplant is the only option for patients that fail medical therapy. *In vivo* genome editing, including base editing, offers the potential for a "one-and-done" therapy for HT1. To date, 95 different disease-causing mutations have been identified in the *FAH* gene. Thus, a single therapeutic base editing strategy to correct the underlying *FAH* mutation in all patients is challenging. As an alternative, a genome editing strategy to inactivate *HPD* offers the potential for a durable, mutation agnostic therapy for all HT1 patients. We previously demonstrated the ability to rescue the lethal mouse model of HT1 via *in vivo* base editing using an adenovirus to deliver a cytosine deaminase and gRNA to introduce a nonsense mutation in the mouse *Hpd* gene. Although encouraging, an adenoviral delivery approach is clinically irrelevant and the gRNA used was specific to the mouse genome. Here, we screen human-specific gRNAs *in vitro* for inactivation of the human *HPD* gene via either cytosine deaminase or adenine deaminase base editing in HEK293 cells and HuH-7 cells, a human hepatoma cell line. This screen identified 4 potential gRNAs, that together with adenine base editor 8.8 (ABE8.8), result in high on-target editing associated with a reduction of HPD

mRNA and protein production. Furthermore, an unbiased off-target analysis assessing 151 potential off-target sites of 2 of the 4 gRNAs found only 3 sites of interest with low-level (<0.5%) off-target editing. To assess the ability of ABE8.8 base editing and candidate gRNAs to rescue the HT1 phenotype *in vivo*, gRNAs specific to the mouse genome that are orthologous to the human gRNAs were designed. ABE8.8 and the orthologous mouse gRNA were delivered systemically to either day of life 1 or adult HT1 mice maintained on NTBC via either a dual AAV8 approach or a lipid nanoparticle (LNP). NTBC was withdrawn two weeks following AAV or LNP injection and mice were monitored for weight gain and survival. At sacrifice, liver function and on-target liver editing was assessed. Control mice consisted of unedited HT1 mice maintained on NTBC and unedited HT1 mice off NTBC. In contrast to unedited HT1 mice which died within 21 days of NTBC removal, edited HT1 neonatal and adult mice demonstrated improved weight gain and survival off of NTBC to the 2 month study end point. At sacrifice, average on-target liver editing was 54% (n=16) and liver function (AST, ALT and Tbili) was normalized in 14 of 16 edited HT1 mice. This study supports adenine base editing using human specific gRNAs and clinically relevant delivery approaches as a mutation agnostic, "one-and-done" therapy for HT1 and positions us to begin IND-enabling studies to seek approval to conduct a clinical trial with a new treatment for HT1.

154 RNA Gene Writers Drive Therapeutically Relevant Levels of Correction of the PAH Gene Responsible for Phenylketonuria in Mouse and Non-Human Primate Models

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Classic phenylketonuria (cPKU) is a genetic disorder caused by autosomal recessive mutations in the phenylalanine hydroxylase gene (*PAH*) resulting in loss of protein function. PAH deficiency leads to increased blood phenylalanine (Phe) levels resulting in toxic accumulation in the brain which can lead to irreversible neurocognitive impairment. The current standard of care for PKU management consists of severe dietary Phe restriction, enzyme replacement therapy and BH4 cofactor supplementation to lower blood Phe levels. Although lowering Phe levels leads to improved neurological performance, problems in executive functioning, motor ability, social skills, and behavior can still occur despite early treatment intervention. As such there is need for new and improved treatment options. We developed RNA Gene Writers to correct the R408W mutation, a common mutation in the human *PAH* gene resulting in a severe form of PKU. RNA Gene Writers are a versatile genome engineering modality that can catalyze the introduction of gene-length DNA sequence as well as site-specific nucleotide changes. Mechanistically, RNA Gene Writers leverage target-primed reverse transcription (TPRT) biochemistry evolved by non-LTR retrotransposon mobile genetic elements, a function that is modular and can be engineered. To correct mutations in the PAH locus we deployed a high throughput screening approach and identified an RNA Gene Writer system comprised of a reverse transcriptase fused to a synthetic RNA-guided Cas nickase and a modified template RNA. We first developed reagents to correct the mutation in the *Pah*^{enu2} PKU mouse model. LNP-RNA delivery of these reagents achieved 40% correction of the *Pah* mutation in the liver of *Pah*^{enu2} mice, resulting in the normalization of Phe levels in the blood and brain. We observed a correlation between *Pah* correction and reduction in plasma Phe levels, and that 10% correction is sufficient to normalize blood Phe levels, in line with previously published work utilizing this same PKU model. A mouse model with the R408W mutation was created by making a transgenic strain with the mouse *Pah* exon 12 replaced by the human *PAH* exon 12 carrying the R408W mutation. We demonstrated an RNA Gene Writer delivered using an LNP achieved up to 35% correction of the R408W mutation in the liver of this novel PKU mouse model, leading to normalized blood Phe levels. Furthermore, we showed our RNA Gene Writing system could install a representative silent mutation in the *PAH* exon 12 of cynomolgus monkeys in up to 45% of liver alleles by LNP delivery, serving as a surrogate model to demonstrate we can achieve therapeutic levels of correction of *PAH* in non-human primates. Taken together, we show that our TPRT-based RNA Gene Writing technology is highly efficient in correcting the mutation causative of PKU in two different mouse models, leading to a phenotypic correction with a single administration of LNP formulated RNAs. Moreover, the introduction of a surrogate mutation in cynomolgus monkeys at efficiencies well above the threshold required for phenotypic rescue in rodent disease models further highlights that the RNA Gene Writing system has the potential to bring therapeutic benefit to patients with PKU after a single administration. Overall, we show that the RNA Gene Writing system can be optimized to achieve high efficiency editing in vitro and in vivo, with a wide-ranging therapeutic potential to treat severe genetic diseases.

155 Development of a Human PCSK9-Targeting Epigenetic Editor with Durable, Near-Complete *In Vivo* Silencing Efficiency

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Epigenetic editing is a powerful new approach to durably silence or activate genes without the risks inherent in editing approaches that use targeted DNA breaks. Epigenetic editors for gene silencing consist of a DNA targeting component fused to a transcriptional repressor

domain and a DNA methyltransferase domain. Transient exposure of the genome to a targeted epigenetic editor durably suppresses gene expression by creating a localized change in the DNA methylation of CpG dinucleotides without cutting, nicking, or altering the DNA sequence. Recent work has demonstrated the utility of epigenetic editors to durably repress gene expression in vitro and in mice. Here, we describe the development of an epigenetic editor targeting human PCSK9 (hPCSK9). Utilizing an epigenetic editor previously reported to drive durable silencing of mouse PCSK9 (mPCSK9) in vivo, we demonstrated that mPCSK9 expression remained durably silenced following a 70% partial hepatectomy, an established model of liver regeneration. To target the hPCSK9 locus, we performed a thorough screen of epigenetic editors across the hPCSK9 locus and identified several that efficiently reduced secreted hPCSK9 levels in immortalized liver cells and primary human hepatocytes. These epigenetic editors were exquisitely specific for silencing hPCSK9, as revealed by deep-read RNA-seq. To examine in vivo activity, we performed a single infusion of an LNP delivering mRNA encoding our optimized epigenetic editing construct targeting hPCSK9, into a transgenic mouse carrying the hPCSK9 genomic locus. We observed near-complete, dose-responsive, durable silencing of the hPCSK9 locus in vivo. Together, our data demonstrate in vivo proof-of-concept for the utility of epigenetic editors to effectively, specifically, and durably silence therapeutic targets in the liver without altering the DNA sequence.

156 Temporally Restricting Cas9 Expression Improves Deletion Frequency in the Liver

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Genomic deletion using CRISPR is a viable strategy to treat disease by eliminating causal mutations including repeat expansions and early termination codon-containing exons. In the absence of guides that target specific alleles, biallelic deletions may be necessary to ensure disease correction. To investigate mono- versus biallelic CRISPR deletion, we used heterozygous Ai14(tdTomato)/Ai6(zsGreen) reporter mice wherein expression of both is induced by deletion of STOP cassette targeted by a common pair of guide RNAs. After AAV8 delivery of Cas9 and gRNAs, hepatocytes exhibited allelic heterogeneity, with mono-allelic deletions (tdTomato+ or zsGreen+) comprising 98% of successful deletions while biallelic deletions (tdTomato+ and zsGreen+) were 2%. Further studies in Ai14/Ai6 mouse embryonic fibroblasts (MEFs) showed unproductive deletions (indels) at gRNA cut sites on the non-expressing fluorescent allele, which infers there was an ineffective biallelic deletion. Because near-simultaneous double-stranded breaks are required for successful deletion prior to indel formation via DNA repair we sought to regulate Cas9 expression, placing it under control of our previously described X^{on} splice cassette (*Monteys 2021 Nature*). In X^{on}, translation is permitted only in the presence of the orally available small molecule inducer LMI070. X^{on} Cas9 was first tested in Ai14/Ai6 MEFs transfected with either an X^{on} or constitutive Cas9/gRNAs-expressing plasmid, and Cas9 translation was induced at 0, 1, 3, 5, or 7 hrs later. Delayed editing (for 5 hrs) resulted in a near 2-fold increase in both the number of overall deletions (5.2% vs 10.2%) and the fraction of deletions that were biallelic (21.2% vs 39.74%) over the constitutively expressed Cas9. To test *in vivo*, Ai14/Ai6 mice were injected with AAV8-gRNAs and either AAV8- X^{on}-Cas9 or AAV8-Cas9 with constitutive expression. Cas9 expression was induced on days 14, 28, and 32, collecting hepatocytes at 56 days post-injection for all groups. While X^{on} -SaCas9 did not alter the overall frequency of deletions compared to the constitutive version (5.3% vs 4.2%), it did significantly improve the fraction of biallelic deletions by 5-fold (0.8% vs 4.02%). In addition to AAV X^{on} based expression, lipid nanoparticles (LNPs) provide a rapid short burst of expression and may further reduce undesired indel generation prior to full deletion. To test this, Ai6 mice were injected with hepatocyte-tropic LNPs at 4mg/kg or 3mg/kg, with a 2:1:1 formulation of Cas9-mRNA : gRNA #1 : gRNA#2. Isolated hepatocytes averaged deletion frequencies of 21% (4mg/kg) and 10% (3mg/kg). Further work is in progress to ascertain the landscape of allelic heterogeneity post-LNP injection, and the potential for re-dosing. Whether the differences between LNP vs AAV X^{on} is due to transgene copies per cell expressing Cas9, or the ramping up of expression over a longer time window is under further exploration. Cumulatively these studies will help inform CRISPR/Cas9 strategies for therapy.

157 Cas-CLOVER Technology Enables Precise Gene Editing and Site-Specific Transgene Insertion in Mouse Liver

Oscar Alvarez, Lucas Homa, Stephanie Kearney, Khalid Hajj, Cindy Negron, Matthew Nitzahn, Arturo Barcenas, Joe Kubicki, Alicia Davis, Mona Connerney, Michael Bennet, Joshua Rychak, Brent Warner, Blair Madison

Poseida Therapeutics - San Diego, CA, San Diego, CA

RNA-guided nucleases such as CRISPR/Cas9 have emerged as powerful molecular tools able to precisely correct genetic diseases by directly editing the genome of patient cells and addressing the root cause of the disease. One major concern for implementing CRISPR/Cas9 for gene therapy is the relatively high frequency of off-target edits. In addition, viral vector delivery of gene editors and therapeutic transgenes can cause both insertional mutagenesis and immunogenic toxicity. Using our high-fidelity, Cas-CLOVER™ technology and proprietary lipid nanoparticles (LNP), we describe a fully non-viral, liver-directed platform. Our approach enables precise knockout editing and site-specific gene insertion of functional transgenes. To deploy this technology, we developed a novel LNP that co-encapsulates multiple nucleic acids in the same particle; this enables the efficient delivery of Cas-CLOVER mRNA and targeting gRNAs to mouse hepatocytes. We demonstrate editing of the mouse *PCSK9* gene to decrease LDL-cholesterol levels as an effective treatment for familial hypercholesterolemia. A single intravenous injection of our optimized Cas-CLOVER LNP achieved high *PCSK9* editing (65% of haploid genomes) and >85% reduction in PCSK9 protein levels. Single nuclei genotyping of liver cells showed PCSK9 editing in 64% of cells (45% biallelic and 19% monoallelic edits). Analysis of 149 computationally predicted off-target sites based on gRNA sequence homology showed no detectable off target editing. In addition, no detectable PCSK9 editing was observed in gonads from male and female mice treated with LNPs comprising Cas-CLOVER RNAs. Blood chemistry

analysis after LNP dosing showed minimal and transient elevation of proinflammatory cytokines and ALT/AST enzymes. Redosing of LNPs was well-tolerated; mice receiving three supra-therapeutic doses of the LNP did not show evidence of liver damage assessed by liver enzyme quantification and histopathological analysis. In a different set of studies designed to test our platform for site-specific gene insertion, we aimed at using Cas-CLOVER to insert a promoterless gene trap cassette encoding a luciferase reporter into a mouse albumin intron. In order to efficiently deliver Cas-CLOVER mRNA, gRNAs and donor DNA to mouse hepatocytes, we developed a second generation LNP that successfully co-encapsulated all nucleic acids in a single particle. With a single LNP injection, we achieved site-specific insertion of the DNA cassette and detected robust luciferase activity in adult, juvenile and neonate mice. Junction PCR analysis confirmed site-specific insertion of the DNA cassette at the albumin intron. In durability studies, luciferase activity persisted for up to three months with minimal loss of signal. Collectively, these results demonstrate the fidelity and tolerability of the LNP platform for targeted transgene insertion using Cas-CLOVER technology in a fully non-viral manner, which underlines the potential of this technology to develop effective therapies for rare diseases.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics II

158 Acoustofluidic Sonoporation-Mediated Gene Delivery Utilizing DNA-Encapsulated Supramolecular Nanoparticles

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Gene therapies where viral or non-viral methods are leveraged for delivering transgene or gene editing constructs to correct the genetic basis for a patient's disease are increasingly being added to the clinical armamentarium. However, the broader clinical expansion of these interventions is, in part, limited by issues with immunogenicity and insertional mutagenesis associated traditional viral vectors, limitations in cargo carrying capacity, and the high costs associated with Good Manufacturing Practice (GMP)-grade vector manufacturing. Non-viral intracellular delivery approaches like electroporation and lipofection are the most common alternative cell therapy manufacturing methods, but still face concerns with efficiency, cytotoxicity, and throughput. To address these concerns, we have developed and tested an acoustofluidic platform that facilitates the uptake of genetic engineering cargoes by applying acoustic waves with high frequency to biophysically manipulate the permeability of cellular membranes transiently as target cells are passed through a microfluidic channel. Here we describe efforts to optimize this methodology for manufacturing chimeric antigen receptor (CAR)-expressing human T cells. We successfully engineered Glypican-3 (GPC3) targeted CAR T cells with ~16% transfection efficiency one week post-sonoporation and stable integration of the CAR (~40% expression) after two weeks by packaging a Sleeping Beauty transposon cassette into supramolecular nanoparticle (SMNP) carriers. The SMNPs enable delivery of larger biomolecular cargoes and more precise dosing when packaging multicomponent payloads. At Day 14, over 50% acoustofluidic-treated cells remained healthy while electroporated cells showed similar viability but much lower transfection efficiency (~25% expression). Our work to improve device consistency and performance probed the kinetics and mechanisms of transient cell/nuclear membrane disruption and repair during acoustofluidic sonoporation. These studies provide insight into the parameters that influence whether delivery is successful in different cell types and how the platform configured and applied to accelerate gene therapy research and clinical translation.

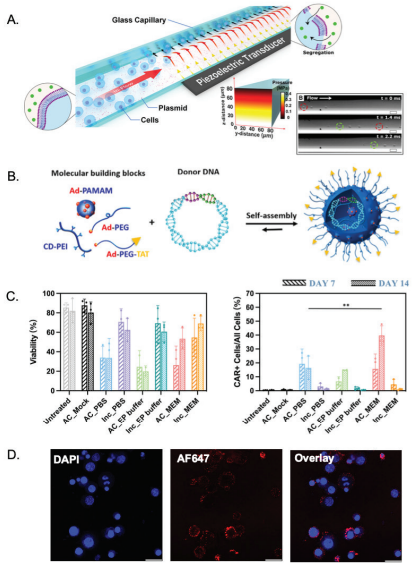

Figure 1. (A) Schematic of the device components and application. (B) A self-assembled synthetic approach for the preparation of plasmid-encapsulated Supramolecular nanoparticles (SMNPs). (C) Cell viability and chimeric antigen receptor (CAR) expression efficiency in human primary T cells in different media 7 days and 14 days post-acoustofluidic treatment. (D) Confocal images of AF647 antibody-stained T cells at Day 14. Scale bar=20 μm.

159 Development of Gold Nanoparticles for *In Vivo* CRISPR Delivery - Improving Nuclease Loading and Stability with Pre-Formed RNP Complexes

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The current state of the art for CRISPR delivery is *ex vivo* electroporation. However, this method is cumbersome and difficult to implement globally. Multiple groups have explored methods for delivering CRISPR components *in vivo*. We previously reported passive delivery of CRISPR cargo and DNA templates into hematopoietic stem and progenitor cells using gold nanoparticles (CRISPR-AuNP) *ex vivo*. We utilized a layer-by-layer assembly, where crRNA for Cas9 or guideRNA (gRNA)

for Cas12a is synthesized with end modification including an 18-oligo ethylene glycol unit-spacer and a terminal thiol linker for conjugation to the AuNP through semi-covalent gold-thiol interactions. For Cas9, crRNA is duplexed with tracrRNA before AuNP addition. Cas9 or Cas12a nuclease protein is then added to form ribonucleoprotein (RNP) complex on the gRNA-AuNP surface. The AuNP-RNP is next encapsulated with positively charged polymer, 2000 MW branched polyethylenimine (PEI), for electrostatic conjugation of negatively charged homology-directed DNA repair template (HDT). Here we sought to analyze this CRISPR-AuNP in more detail to optimize functionality. We evaluated RNP loading and functionality following CRISPR-AuNP assembly by releasing cargo with β-mercaptoethanol (BME) and performing sodium dodecyl sulfate polyacrylamide gel electrophoresis. This showed no detectable Cas9 nuclease protein, but detectable Cas12a (Fig. 1a), possibly explaining our prior results showing superior performance of Cas12a in this system. A quantitative Qubit protein assay demonstrated CRISPR-AuNP nuclease loading equivalent to 5 ± 6 Cas9 nuclease per particle (n=2) and 16±1 Cas12a nuclease per particle (n=2). Moreover, we observed loss of tracrRNA when Cas9 nuclease was added to gRNA-AuNP, possibly due to steric crowding as gRNA is loaded in excess to stabilize and prevent AuNP aggregation. We hypothesized that pre-forming RNP complexes could ensure equivalent gRNA:nuclease ratios and increase the number of active RNP per AuNP. However, low pH conditions may compromise nuclease function and altered loading capacity could reduce AuNP stability. To test, RNP were pre-formed under standard conditions with end-modified gRNA and then added to AuNP in the presence of citrate at pH 3.7 to induce gold-thiol interaction. We show stable RNP binding to AuNP with preformed Cas12a or Cas9 RNP complexes with higher nuclease loading: 144.5±4.5 Cas9 nuclease (n=2) and 87±13 Cas12a (n=2) nuclease per particle with functional in-tubo cutting activity (Fig. 1b), despite low pH conditions. However, PEI addition to preformed RNP-AuNP caused aggregation, suggesting charge instability. Testing different PEI conjugates identified a 2000 MW polyethylene glycol (PEG)-conjugated PEI stabilized preformed RNP-AuNP and permitted loading of HDT between 100bp and 2.1kb in length. These improved CRISPR-AuNP displayed a hydrodynamic diameter of 75.75±12.59/70.06±1.46 nm and polydispersity index of 0.16±0.1/0.20±0.01 for Cas9/Cas12a AuNP respectively (Fig. 1c). The preformed AuNP were tested *in vivo* as described in abstract 2023-A-1264-ASGCT.

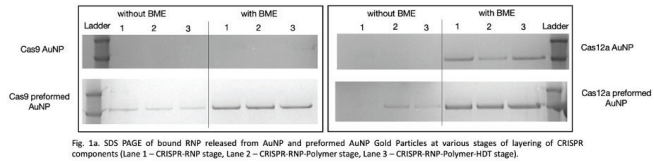

Fig. 1a. SDS PAGE of bound RNP released from AuNP and preformed AuNP Gold Particles at various stages of layering of CRISPR components (Lane 1 – CRISPR-RNP stage, Lane 2 – CRISPR-RNP-Polymer stage, Lane 3 – CRISPR-RNP-Polymer-HDT stage).

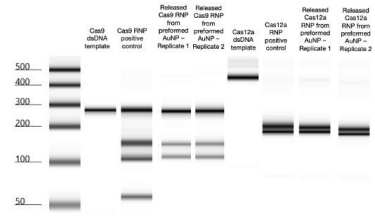

Fig. 1b. In-tubo cutting activity showing functional nuclease activity in released RNP from preformed-AuNP using BME

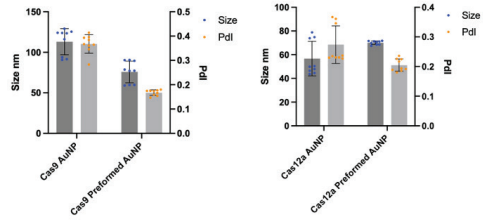

Fig. 1c. AuNP and preformed AuNP size and polydispersity index (PdI) characteristics

160 Molecular Chameleon Carriers for Nucleic Acid Delivery: The Sweet Spot between Lipoplexes and Polyplexes

Sophie Thalmayr, Melina Grau, Lun Peng, Jana Pöhmerer, Ulrich Wilk, Paul Folda, Mina Yazdi, Eric Weidinger, Tobias Burghardt, Miriam Höhn, Ernst Wagner, Simone Berger

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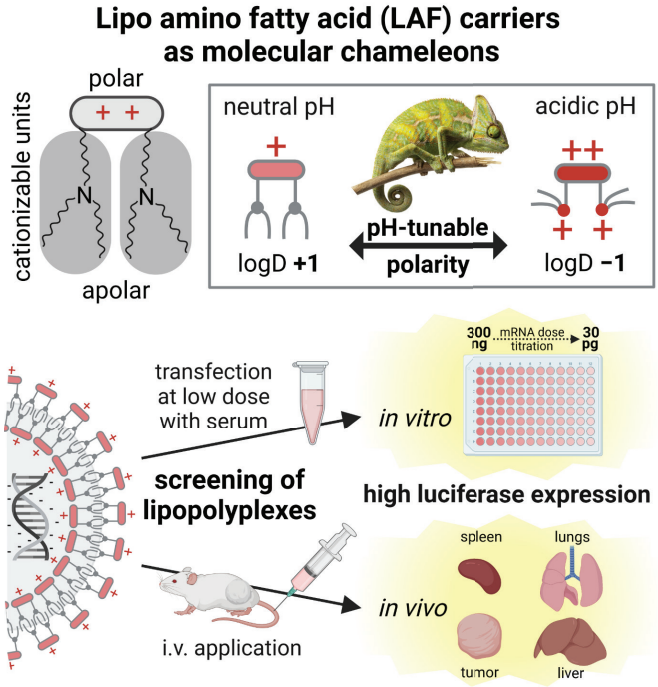
Taking advantage of effective intracellular delivery mechanisms of cationizable lipids and cationic polymers, highly potent nucleic acid carriers were generated by combining at least two novel lipo amino fatty acids (LAFs) as cationizable lipidic motifs with well-known polar cationizable aminoethylene units into sequence-defined molecules. The pH-dependent tunable polarity of the LAF was successfully implemented by a central tertiary amine, which disrupts the hydrophobic character once protonated, resulting in pH-dependent structural and physical changes, as evidenced by drastic changes in the logarithmic distribution logD (octanol/water) from around +1 (pH 7.4) to -1 (pH 5.5). This "molecular chameleon character" turned out to be advantageous for dynamic cargo delivery via lipopolyplexes. Chemical evolution by screening of different topologies (blocks, bundles, T-shapes, and U-shapes), different LAF/aminoethylene ratios, and different LAFs identified tailor-made carriers for various nucleic acid cargos, i.e., plasmid DNA (pDNA), messenger RNA (mRNA), and small-interfering RNA (siRNA). In the case of pDNA and mRNA, bundles with short LAF hydrocarbon chains as well as U-shapes were favorable. For siRNA, the U-shape topology was most potent. Noteworthy, the efficiency of the best performers was up to several hundred-fold higher compared to previous carrier generations and

characterized by very fast transfection kinetics. mRNA lipopolyplexes maintained high transfection activity in cell culture even in the presence of 90% serum at extremely low nucleic acid dosage of 30 picogram (around 20 nanoparticles per cell; 10,000-fold lower dose than in standard transfection), and thus are almost as potent as viral vectors. Finally, they showed great in vivo performance with high expression levels especially in spleen, tumor (N2a neuroblastoma tumor model), lungs, and liver upon intravenous administration of 1-3 microgram luciferase-encoding mRNA in mice.



161 Environmentally-Sensitive Polymer-Based Nanoparticles for Intravitreal Gene Delivery

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An adeno-associated virus (AAV)-based gene therapy product has recently received FDA approval for treating a rare retinal disorder via the subretinal route. However, AAV has several shortcomings, including low packaging capacity, therapy-inactivating immunogenicity, and prohibitive cost. Moreover, subretinal injection is highly invasive, causes ocular damage, and limits spatial therapeutic coverage to near the injection site. Thus, a cost-effective means to provide safe, widespread, and efficacious retinal gene therapy is needed to meet the ever-increasing need. Intravitreal injection of non-viral gene delivery nanoparticles (NPs), devoid of many of the aforementioned limitations, may constitute an attractive alternative. However, intravitreally injected NPs must overcome two key extracellular barriers, the vitreous gel and inner limiting membrane (ILM), to reach the retina. We thus developed a polymer-based gene delivery NP platform capable of penetrating these barriers after intravitreal injection to mediate efficient retinal transgene expression. Specifically, we *de novo* synthesized a polyethylene glycol (PEG)-conjugated bioreducible cationic polymer, poly(disulfide amine) (PDSA), that compacts plasmids to form NPs while promoting the intracellular release of payloads. Of note, PEG endows otherwise cationic NPs with non-adhesive surfaces to minimize adhesive electrostatic interactions within the negatively charged vitreous gel and ILM, thereby facilitating their permeation through these barriers. We first confirmed the efficient compaction of reporter luciferase or ZsGreen1 plasmids by PDSA or PEG-PDSA (**A**) and the formation of NPs possessing hydrodynamic diameters of < 80 nm. We then showed that PDSA provided significantly greater *in vitro* reporter transgene expression compared to other lead synthetic materials with an excellent safety profile in human retinal pigment epithelial (ARPE-19) cells (**B**-**D**). Multiple particle tracking analysis revealed that PEG-PDSA NPs, unlike PDSA NPs, efficiently penetrated rabbit vitreous (**E, F**). Using a bovine vitreoretinal explant model and size-controlled polystyrene beads, we discovered that NPs sized ≤ 100 nm can efficiently penetrate the ILM only if the particle surfaces are shielded with PEG (**G**), providing design criteria for engineering ILM-penetrating NPs. Accordingly, we found that small and non-adhesive PEG-PDSA NPs efficiently penetrated the ILM (**H**) and mediated retinal transgene expression (**I**) while PDSA NPs were unable to do so. Finally, we demonstrated that PEG-PDSA NPs, due to their unique ability to penetrate both vitreous gel and ILM, provided robust and widespread retinal reporter transgene expression in mouse eyes following intravitreal injection (**J**-**L**). PEG-PDSA NPs, upon their further development, may serve as a broadly applicable delivery platform for retinal gene therapy.

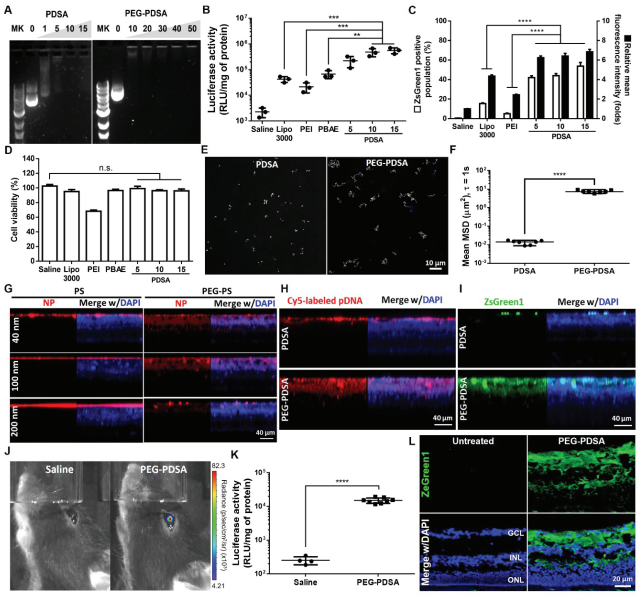

Figure 1. PEG-PDSA NPs are capable of efficiently penetrating vitreous gel and ILM to mediate robust and widespread retinal transgene expression following intravitreal injection. **(A)** Gel electrophoretic migration assay demonstrating full compaction of plasmids by PDSA or PEG-PDSA at varying polymer to plasmid ratios. **(B,C)** *In vitro* transfection efficiency and **(D)** cell viability of PDSA NPs in ARPE-19 cells. Transfection efficiency was determined by **(B)** luciferase activity and **(C)** ZsGreen1 expression. **(E)** Representative trajectories of PDSA and PEG-PDSA NPs in rabbit vitreous gel. **(F)** Mean square displacement (MSD) at a time scale (τ) of 1 second of PDSA and PEG-PDSA NPs. The MSD value is directly proportional to the particle diffusion rate. **(G)** Representative vertical images of bovine vitreoretinal (VR) explants treated with 40, 100, or 200 nm polystyrene (PS) or PEG-PS NPs. **(H, I)** Representative vertical images of bovine VR explants treated with PDSA or PEG-PDSA NPs. NPs were prepared to carry **(H)** Cy5-labeled plasmids or **(I)** ZsGreen1-expressing plasmids for microscopic observation of NP penetration (red) or transgene expression (green), respectively. **(J)** Representative IVIS images of a C57BL/6 mouse eye intravitreally treated with PEG-PDSA NPs, in comparison to a saline-treated eye. **(K)** *In vivo* transfection efficiency determined by tissue homogenate-based luciferase assay. **(L)** Representative confocal images showing ZsGreen1 transgene expression (green) in a C57BL/6 mouse eye intravitreally treated with PEG-PDSA NP. Blue staining represents cell nuclei. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. n.s.: no significance, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

162 Altered Localization of Plasmid DNA within the Nucleus Affects Gene Expression

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Upon plasmid entry into the nucleus, most assume that gene delivery has been completed, and that transgene expression levels are mostly dependent on the number of plasmids that have reached the nucleus. However, we and others have shown that robust redistribution of plasmids occurs within the nucleus. We show here that this movement can affect gene expression levels. We have previously reported that plasmids redistribute within the nucleus in a transcription-dependent manner; plasmids carrying Pol I promoters and expressing Pol I transcripts migrate to the nucleolus while plasmids carrying Pol II promoters and expressing mRNAs colocalize with nuclear speckles (Pol II promoter transcription factories). Plasmids lacking any eukaryotic promoter or that are transcriptionally inactive fail to redistribute and show diffuse staining throughout the nucleoplasm. How Pol III constructs (expressing tRNAs and shRNAs) localize within the nucleus is unknown. We microinjected cell nuclei of A549 epithelial cells with plasmids carrying either U6 or tRNA Pol III promoters, and used fluorescent in situ hybridization (FISH) to visualize the nuclear plasmid spatial location. Unlike Pol I or Pol II plasmids, Pol III promoter-containing plasmids redistribute into small nuclear foci distinct from nuclear speckles. Interestingly, a single plasmid carrying both Pol II and Pol III promoter-gene cassettes redistributes into a pattern unlike those of plasmids carrying either single promoter-gene cassette only. Inhibition of Pol II transcriptional activity causes the dual promoter-containing plasmid to be redistributed into foci that are very similar to those seen with Pol III plasmids. Similarly, Pol III transcription inhibition caused the dual promoter-containing plasmid to show more Pol II promoter-plasmid redistribution. This altered distribution of the dual promoter class plasmid required the two different classes of promoter to be in cis on the same plasmid. To determine the functional consequences of this subnuclear mislocalization, we created isogenic plasmids containing a Pol III-shRNA cassette and/or a Pol II-GFP cassette together or individually. We microinjected the same copy number of each plasmid, alone or in combination, into the nuclei of cells, and evaluated GFP expression over time. When the Pol II-GFP plasmid was injected alone or together with the separate Pol III plasmid, robust GFP expression was detected with no difference in intensity or percentage of cells expressing GFP. However, when the dual promoter plasmid (II and III) was injected, weaker GFP expression was detected and fewer cells expressed GFP. These results demonstrate that the location of plasmids within the nucleus can affect their expression level. Taken together, these results point to the need to understand and optimize the intracellular trafficking events of plasmids both in the cytoplasm and nucleus in order to maximize nonviral gene transfer.

163 Neuroprotection by Optical Delivery of Therapeutic PEDF Gene into Retina

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Introduction: Vision loss and blindness associated with dysfunction/degeneration of retinal ganglion cells (RGCs, in Glaucoma), retinal pigment epithelium (RPE, AMD), or photoreceptors (Retinal degenerative diseases), are caused by genetic and/or epigenetic changes including oxidative stress (in dry-AMD, DR: diabetic retinopathy). Pigment epithelium-derived factor (PEDF) has been found to act as a neuroprotector for a wide variety of neurons including retinal cells and has potential to also act as anti-fibrotic/angiogenic agent by modulating oxidative stress and inflammatory responses. However, gene delivery using viral vectors is associated with inflammatory and immune response. Therefore, we have developed an optical coherence tomography (OCT) guided ultrafast near-infrared (NIR) laser based non-viral delivery method for spatially localized delivery of PEDF-gene into retina. **Method**: For in-vivo laser delivery into RGCs, the PEDF plasmids were injected intravitreally, and the retina was irradiated by OCT-guided ultrafast NIR laser in wild type mice. The safety of laser gene delivery to targeted retinal cells was evaluated by multiple measures including electrophysiology (ERG), intra-ocular pressure (IOP), OCT imaging, Visual evoked potential (VEP) and immunohistochemistry. Acute injury caused by KCL/NMDA insults were used to evaluate neuroprotection efficacy of laser delivered PEDF. To evaluate anti-angiogenic effect of non-viral PEDF gene therapy, laser induced choroidal neovascularization (CNV animal model of wet-AMD) was used. **Results**: In-vivo ultrafast NIR laser-based delivery of PEDF genes was confirmed by expression of fluorescence reporter in targeted cell layer and treated regions. Animals with laser delivered PEDF exhibited significantly higher ERG amplitude compared to non-treated group. Significant retention of VEP amplitude in PEDF-treated mice was observed in contrast to non-treated group demonstrating the neuroprotective effect. Further, RGC survival was higher in the PEDF-treated group. In addition, our study on CNV mouse model demonstrated anti-angiogenic effect of PEDF. Structural analysis with OCT image shows that PEDF treated mouse sustained less damage compared to non-treated group subjected to the same laser irradiation which corroborated by isolectin-IB4 staining of the retinal flat mount. **Conclusion**: The non-viral laser delivery approach provides efficient, targeted delivery of therapeutic PEDF encoding gene into spatially targeted retinal cells for neuroprotection and anti-angiogencity. We have identified/engineered PEDF mutants which has enhanced neuroprotective/anti-angiogenic properties with long lasting expression coupled with a non-viral laser delivery to address the critical need for neuroprotective/anti-angiogenic therapy of multiple complex ocular disorders.

164 Development of a Monoparticle-Based CRISPR Gene Editing and Intraparenchymal Delivery System for Neurological Applications

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Introduction. CRISPR-based gene editing within the central nervous system (CNS) has the potential to revolutionize the treatment of neurological disorders, including currently-incurable monogenic neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's diseases. However, developing CRISPR-based therapeutics for the CNS has been challenging, due in large part to drug delivery. Two key obstacles need to be overcome before gene editing in the human brain is feasible: (1) efficient and safe delivery of Cas9 and gRNA into neurons, and (2) establishment of strategies that enable targeted brain regions to be efficiently edited. The goal of this study is to develop formulations of Cas9 ribonucleoprotein (RNP) enzymes that can edit large volumes of targeted brain structures after intracranial injection. Our team - a collaboration of the Wilson, Murthy, and Bankiewicz laboratories - has developed a non-viral delivery system involving peptide-mediated intracellular delivery of a RNP complex administered via intrastriatal injection using convection-enhanced delivery (CED). This approach offers several advantages, including ease of production, transient CRISPR activity, and robust tissue distribution enabled by exceptionally small particle size. **Material and Methods.** We have developed a nuclear localization signal (NLS)-rich Cas9 nuclease protein combined with sgRNAs resulting in small particles (≤20nm), or "monoparticles." A series of different monoparticle formulations were developed that contained NLS-rich Cas9-RNPs and amphipathic peptides promoting intracellular delivery. A pair of gRNAs were used to target the cassette repressing tdTomato fluorescent reporter expression in transgenic Ai9 reporter mice. The various monoparticle (RNP:peptide) formulations were screened using a cell-based *in vitro* system. Lead Cas9 formulations were then assessed *in vivo* by administration into the striatum of Ai9 mice via CED. Three weeks following infusion, animals were euthanized and their brains were processed for immunohistological analysis of distribution and efficiency of neuronal genomic editing within the target structure. **Results and Conclusion.** Our best performing RNP:peptide monoparticle formulation exhibited an editing efficiency of approximately 82% of neurons within a coverage area involving over 33% of the striatum in Ai9 mice. This optimal formulation was then combined with sgRNAs targeting the gene expressing green fluorescent protein in transgenic GFP mice, where we observed knockdown of GFP expression in 76% of the neurons in the target area. We conclude that this monoparticle-based CRISPR reagent delivery system is efficacious in genomic editing of CNS neurons following intracranial delivery to target brain regions. We are currently advancing this technology to large animals utilizing MRI-guided CED for intraparenchymal delivery. This approach holds the promise to accelerate successful translation of CRISPR-Cas9 to the clinic for the treatment of brain diseases.

Neurological Gene Therapies in Advanced Stages of Clinical Translation

165 Lowering of Toxic Dipeptide Proteins and Phenotype Rescue in an ALS Mouse Model Treated with AAV-miQURE® Targeting the Repeat Expansion-Containing C9orf72 Transcripts

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Amyotrophic lateral sclerosis (ALS) is a progressive, fatal, neurodegenerative disorder that affects the upper and lower motor neurons in the brain and corticospinal tract leading to eventual muscle atrophy and paralysis. The most common genetic cause of familial ALS is a hexanucleotide (GGGGCC) repeat expansion in the first intron of the chromosome 9 open reading frame 72 (*C9ORF72*) gene. The bi-directionally transcribed, expanded, repeat-containing C9orf72 mRNA causes cellular toxicity due to RNA foci and dipeptide repeats (DPRs) production, both of which are the pathogenetic hallmarks of the disease. The *C9ORF72* gene contains 11 exons and is transcribed into three major transcripts variant 1 (V1), variant 2 (V2) and variant 3 (V3). The repeat expansion is located between exons 1a and 1b, and it is situated in the first intron of the toxic variants V1 and V3 and in the promoter region of the healthy variant V2. We developed therapeutic microRNAs (miRNAs) using our miQURE® platform to selectively silence the repeat-containing C9orf72 transcripts. The most potent therapeutic miRNAs (miC9Os), identified *in vitro,* have been tested for their efficacy in lowering mutant intronic C9orf72 mRNAs in two ALS mouse models. In the transgenic ALS mouse model (Tg(C9orf72_3) line 112), we have shown that intra-striatal administration of AAV-miC9O leads to significant lowering of the human intronic C9orf72 mRNAs, without affecting healthy (V2) human and total C9orf72 mRNA expression. Next we assessed functional improvement in the AAV9-(G4C2)149 repeats-induced ALS mouse model which exhibits motor deficits, hyperactivity, learning and memory dysfunction starting at 26 weeks of age. Intra-striatal administration of AAV-miC9O in the AAV9-(G4C2)149 repeats-induced mouse model resulted in a dose-dependent knockdown of mutant intronic C9orf72 mRNAs at 8 weeks post-AAV-miC9O dosing. In accordance with the strong intronic C9orf72 mRNA lowering (up to 75%) in the striatum and cortex, a significant lowering of DPRs at 18 weeks after intra-striatal administration of AAV-miC9O was measured. The behavioral assessment performed at 26 weeks of age, 18 weeks post-AAV dosing, revealed that higher proportion of mice that received a high dose AAV-miC9O retained muscle strength compared to negative control. In the fear conditioning paradigm, AAV-miC9O treatment groups showed higher immobility time during exposure to the conditioned-context compared to negative control, suggesting rescued memory of fear and cognitive function in the treatment groups. In conclusion, we have shown strong and sustained lowering of intronic C9orf72 mRNAs and

rescue of ALS phenotype in mice post AAV treatment. Current results support further investigation of AAV-miC9O distribution, efficacy, tolerability, and safety of AAV-miC9Os in small and larger animals. *miQURE is a registered trademark in the US and other jurisdictions.*

166 Long-Term Efficacy of Gene Therapy for AADC Deficiency, Including Patients with a Moderate Phenotype

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Aromatic l-amino acid decarboxylase (AADC) deficiency presents with dystonia, oculogyric crisis, loss of voluntary movements, developmental delay, and autonomic symptoms caused by defects in the *dopa decarboxylase* (*DDC*) gene. Here we report long-term gene therapy results for AADC deficiency, focusing especially on patients with a moderate phenotype. Ten patients (8 severe and two moderate types) were enrolled. The patients received bilateral intraputaminal injections of an adeno-associated virus type 2 vector, harboring *DDC* (2×10^{11} vector genomes), through stereotactic brain surgery. They were then followed up for a period lasting from 6 months to 7 years. Positron emission tomography (PET) imaging with a specific AADC tracer revealed that increased uptake persisted for five years after treatment. In all patients, truncal and limb dystonia attacks disappeared. Oculogyric crisis decreased markedly. In severe patients, it persisted but only resulted in mild eye deviation. All severe patients could not move voluntarily before the treatment. After the treatment, four severe patients could walk with a walker, and one severe patient could swim (video presentation). Two moderate patients who could walk with support before the treatment began to walk and run independently. The Alberta Infant Motor Scale (AIMS) scores increased to more than 20 points in patients treated at a younger age and to more than 10 points in patients treated at an older age. In one moderate patient who was treated at four years of age, her cognitive function improved so that she could begin to make conversation, and her developmental quotient scores increased from 40 to 80. In addition, her eye movements when following objects improved, as revealed by the eye tracker. PET and MRI imaging analysis revealed that the highly transduced putaminal area connecting to the prefrontal cortex was associated with improved motor performance at 6M post-treatment, suggesting that putaminal dopamine promotes the development of an immature motor control system. In the other patient with a moderate phenotype who was treated at 12 years of age, his motor ability and cognitive function improved mildly. Cognitive and motor function improved in patients with moderate phenotypes. However, the efficacy of AADC gene therapy is limited, even in moderate patients, if treatment is delayed till they are older. In moderate patients, residual enzyme activity can create dopamine to induce neuronal network formation at some level, with the accelerated improvement of the neural network after gene therapy if treatment is provided at a younger age.

167 Intraparenchymal Convection Enhanced Delivery of AAV in Sheep to Treat Mucopolysaccharidosis IIIC

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Mucopolysaccharidosis IIIC (MPSIIIC) is one of four Sanfilippo diseases sharing clinical signs and symptoms of severe cognitive and later motor decline and shortened life span. Unlike most other lysosomal diseases the missing enzyme, heparan sulfate acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT), is bound to the lysosomal membrane and thus cannot cross the blood-brain barrier or diffuse between cells. We have previously demonstrated behavioural, biochemical and pathological correction of this disease in the mouse model of MPSIIIC using an Adeno-Associated Vector (AAV) delivering recombinant human HGSNAT from two intraparenchymal injections into the brain using an AAV-2 derived AAV truetype (AAV-TT) serotype with improved distribution over AAV9. Current Adeno-Associated Vector (AAV) gene therapy delivery routes are sub-optimal for effective neuronal delivery in the entire human brain, which is essential in diseases characterised by global neurological pathology such as Mucopolysaccharidosis (MPS) IIIC. We describe AAV delivery using Brainlab targeted catheters or Hamilton syringes for convection enhanced delivery (CED) in sheep, designed to reduce proximal vector expression and improve spread in intraparenchymal injections. Using AAV-GFP, we found that Brainlab cranial navigation is optimal for gene therapy, although Hamilton syringes gave improved distribution over catheters, despite higher doses and titres of vector used in catheter delivery. Intraparenchymal CED gives better distribution compared to intracerebroventricular delivery for the same vector dose. We demonstrate that we can effectively deliver functional HGSNAT enzyme in 24-37% of a 140g gyrencephalic sheep brain using AAV9-HGSNAT in only 3 injections in one hemisphere. AAV serotype may also be important, as AAVTT-GFP displayed moderately better transduction compared to AAV9-GFP but both serotypes almost exclusively transduced neurons via this route. In addition, the presence of pre-existing IgG antibodies in serum does not seem to affect AAV transduction in the brain. Despite variabilities in vector purification, volume and titre, we found the primary attribute for efficient brain delivery is catheter design. These data help to inform a future potential clinical trial for MPSIIIC.

168 Subacute Liver Injury in Two Young Infants Following Gene Replacement Therapy for Spinal Muscular Atrophy

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Introduction: Spinal muscular atrophy (SMA) is a neurodegenerative disorder resulting from irreversible loss of anterior horn cells secondary to homozygous mutations in the survival motor neuron gene, *SMN1*. Gene replacement therapy using an AAV9 vector containing an *SMN1* gene construct, onasemnogene abeparvovec-xioi (OA), was approved by the U.S. Food and Drug Administration in May, 2019. Subacute mild elevation of liver function tests following infusion of OA has been shown to be a relatively common adverse event. Additionally, there have been case reports of liver failure following administration of this therapy. The current recommendations from the drug manufacturer advise systemic corticosteroids to attenuate immune reaction and transaminitis. However, there is a paucity of data regarding management of liver injury refractory to protocol steroids. We report 2 cases of subacute severe elevation of liver function tests >10-20 times the upper limit of normal following OA administration in young infants less than 4 weeks old. **Cases:** In case 1, patient received OA on day of life 19. At 4 weeks post gene transfer, she was admitted for inpatient management of acute liver injury. Liver biopsy was completed which showed grade 2/4 hepatitis. Over the next 3 weeks she had worsening liver injury despite escalating doses of intravenous methylprednisolone (IVMP) and a 3-day course of anakinra. After high dose IVMP at 30mg/kg and concurrent tacrolimus, liver enzymes began to improve. In case 2, patient received OA on day of life 13. At 4 weeks post gene transfer, she was admitted for inpatient management of acute liver injury. She exhibited improvement on a 3-day course of IVMP at 10mg/kg, however required readmission at 6 weeks post gene transfer due to worsening liver injury. At that time, she underwent liver biopsy which showed grade 3/4 chronic hepatitis, inflammatory cells (predominantly CD3 positive T cells), and stage 2/4 fibrosis. A second course of high dose IVMP at 10mg/kg resulted in improvement of liver enzymes. Neither case met criteria for liver failure with normal international normalized ratio, and in both cases, evaluation for other causes of liver disease was unrevealing. Inflammatory markers were elevated and followed similar trends to liver markers. Corticosteroids were continued for both patients for four months post gene therapy. **Conclusion:** We present 2 cases of liver injury with moderate to severe elevations in liver function tests in young infants with SMA following treatment with OA at less than 4 weeks old. Onset of liver injury at 4 weeks post gene transfer with laboratory evidence of systemic inflammation and biopsy reflective of T cell mediated inflammation is consistent with prior case reports. Safety of OA administration in young infants (<1 month of age) has not been characterized separately from other pediatric patient populations, and established standards of care for management of immune response as manifested by liver injury refractory to protocol steroids is an area of clinical need. High doses of corticosteroids up to 30mg/kg daily may be required, and there may be indication for adjunct immunosuppressive treatment in addition to corticosteroids. These questions are pertinent to the management of SMA patients receiving OA but also have broader implications for gene therapy using AAV.

169 Efficacy and Safety of a Novel *FXN* Gene Therapy (AVB-202) for the Treatment of Friedreich's Ataxia

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Friedreich's ataxia (FA), the most common form of hereditary ataxia, is an autosomal recessive disorder caused by mutations in the frataxin (*FXN*) gene, which encodes a mitochondrial protein important for energy production. FA is a systemic disorder characterized by cardiomyopathy, gait ataxia, and sensory loss among other central nervous system (CNS) manifestations. The main cause of death in the majority of FA patients is cardiomyopathy. AVB-202, a novel AAV9 gene therapy product expressing *FXN* under the control of a CBA promoter, is intended to restore functional levels of frataxin across disease-relevant tissues using a dual route of administration (intravenous and intrathecal) with the goal of preventing progression or reversing cardiac and CNS manifestations of FA. Development of AVB-202 includes assessments in relevant animal models of cardiac and neurologic manifestations of the disease and long-term safety. Results from cardiac efficacy and long-term safety studies will be presented. A preclinical study was conducted to evaluate the efficacy of AVB-202 using a conditional knock out mouse model of FA that develops progressive cardiomyopathy and early mortality (referred to as mutant [MUT]). Untreated MUT mice exhibit abnormalities on echocardiogram as early as 6 weeks of age and mortality by 9-11 weeks of age. Mice received a single intravenous injection of AVB-202 at varying doses at 3 weeks of age. Cardiac function was assessed at regular intervals using echocardiography. Additionally, biochemical and histologic assessments were performed on cardiac tissues. Dose-dependent extension of lifespan and improvements in cardiac abnormalities occurred with AVB-202 treatment of MUT mice. Dose-dependent increases in frataxin protein were also noted in cardiac tissue. Furthermore, activity of a biomarker of mitochondrial function, the mitochondrial enzyme succinyl dehydrogenase, was also improved in cardiac tissue. Together, these results support that AVB-202 restores functional levels of frataxin in cardiac tissue and ameliorates manifestations of FA-associated cardiomyopathy. To assess long-term safety, non-human primates (NHP) received a single dose of AVB-202 either intravenously, intrathecally, or by dual route of administration at multiple dose levels. Animals were monitored for 6 months and biochemical and histologic assessments were performed at the end of the study. AVB-202 was well tolerated with a favorable safety profile across multiple doses and routes of administration. Robust expression of frataxin was noted in key FA target tissues, including the heart and CNS. The strong positive effects on survival and cardiac function in

a cardiac mouse model of FA, as well as favorable safety profile and robust transgene expression across multiple organs in NHPs, support continued development of AVB-202.

170 Preclinical Safety Assessment of NGN-401, a Clinical-Stage Gene Therapy Product for Rett Syndrome

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Background: Rett syndrome is a severe neurological disorder caused by loss-of-function variants in the X-linked *MECP2* gene. Females with Rett syndrome exhibit a mosaic pattern of expression whereby approximately half of cells exhibit substantial deficiency in functional MeCP2. Conversely, individuals with duplication at the *MECP2* locus also experience severe neurological disease, indicating that the gene is highly dosage sensitive and creating challenges for conventional gene therapy. NGN-401 is an investigational AAV9 gene therapy for Rett syndrome designed to provide full length and fully functional MeCP2 protein within a narrow expression range via a self-regulating miRNA circuit, termed EXACT. In preclinical studies and in the clinic, NGN-401 is delivered via intracerebroventricular administration. **Results:** Studies in male *Mecp2* knockout mice, a model that shares phenotypic features of the human disease, have shown that delivery of NGN-401 extends survival and ameliorates disease phenotypes. The safety of NGN-401 was evaluated in female mice heterozygous for the null allele and mirroring the mosaic MeCP2 expression patterns observed in female Rett syndrome subjects. Mice treated with 0.9-2.7X the clinical dose of NGN-401 had no observable in-life toxicity through 26 weeks of age. At the maximum feasible dose (6.4x clinical dose), a minimal in-life hindlimb phenotype was observed, with no progression. Moreover, no NGN-401 related histopathological findings were observed at the 2-month interim sacrifice in any of the tissues evaluated. In contrast, even at the lowest dose evaluated, severe toxicity was observed following delivery of an AAV9 vector carrying the *MECP2* transgene without EXACT regulation, requiring sacrifice by ~3 weeks of age. Vector genome levels were broadly similar between vectors, but MeCP2 expression was lower with NGN-401 administration. The safety of NGN-401 was further evaluated in nonhuman primates expressing physiological levels of MeCP2 in all cells and thus representing a high bar for evaluation of safety. In a 6-month GLP-compliant study in juvenile female nonhuman primates, NGN-401 was well-tolerated with a 4.1-fold safety margin over the clinical dose. In a separate comparative study with unregulated vector, NGN-401 was consistently well-tolerated, and biodistribution and mRNA expression in key areas of the brain involved in Rett syndrome pathogenesis was confirmed. In contrast, delivery of a conventional unregulated *MECP2* vector led to an adverse effect on sural nerve conduction, highlighting a difference in safety profile between NGN-401 and a vector without EXACT regulation. Comparison of transgene expression following delivery of both vectors demonstrated the ability of the EXACT circuit to constrain and reduce variability in expression levels in the central nervous system. **Conclusion:** Overall, the totality of data generated in multiple preclinical models demonstrated that NGN-401 allowed for MeCP2 expression that provided therapeutic benefit while mitigating the risk of overexpression toxicity, supporting FDA clearance to initiate a first-in-human pediatric clinical study.

171 Comparison of Dorsal Root Ganglion Toxicity in the Development of AAV9/MFSD8 and AAV9/AP4M1 Gene Therapies for CLN7 and SPG50

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Adeno-associated viral (AAV) vector delivery has emerged as the dominant vehicle to deliver genes to patients affected with genetic disorders to permanently treat these disorders with a single treatment. To date, five AAV-based gene therapy drugs have been approved by the US Food and Drug Administration or the European Medicines Agency. Although AAV in general has a favorable safety profile, it has been reported recently that AAV, especially in high dose, can induce immune cell infiltration and neuron degeneration in dorsal root ganglion (DRG). During the development of intra-CSF (cerebrospinal fluid) AAV9/*MFSD8* (*JeT-MFSD8-SV40pA)* and AAV9/*AP4M1 (UsP-AP4M1-BGHpA)* gene therapies to treat CLN7 batten disease and spastic paraplegia 50 (SPG50) respectively, we evaluated the DRG toxicity following gene transfer in WT (wild type) animals, including 12-month long-term *non-GLP* studies in C57BL/6J mice, 3-month short-term *GLP* studies in Sprague Dawley rats, and 3-month short-term studies in non-human primates (NHPs). In the mouse studies, we treated the mice by lumbar intrathecal (IT) administration at 6-8 weeks of age with AAV9/*MFSD8* (0.447E11, 1.48E11, 4.47E11, or 9.5E11 vg/mouse) or AAV9/*AP4M1* (1.25E11 or 5E11 vg/mouse) and did not note any DRG toxicity up to12-months post administration. In the rat studies, we treated the rats IT at 7-8 weeks of age with AAV9/*MFSD8* (0.5E12, 2E12, or 6E12 vg/rat) or AAV9/*AP4M1* (0.36E12, 1.1E12, or 3.3E12 vg/rat) and noted in the lumbar DRG no neuronal and axonal degeneration with AAV9/*MFSD8* up to 6E12 vg/rat, but minimal to mild neuronal and axonal degeneration in both male and female rats with ≥ 1.1E12 vg/rat of AAV9/*AP4M1*. The degeneration in the rats treated with AAV9/*AP4M1* was apparent at day 8, was worse at day 29, and was less severe at day 91 post administration. Similar results were observed in the NHP studies, where we treated 1) naïve Japanese macaques (*Macaca fuscata*) at 3-4 years of age ICM (intracisternal magna) with AAV9/*MFSD8* (1E14 vg/monkey) or 2) Cynomolgus monkeys at 2-4 years of age IT with AAV9/*AP4M1* (0.84E14, or 1.68E14 vg/monkey), along with immunosuppression regimen (sirolimus and methylprednisolone). Once again, we noted in the lumbar DRG no neuronal degeneration with AAV9/*MFSD8*, but minimal neuronal degeneration in both male and female monkeys at days 94 post administration of AAV9/*AP4M1* at 1.68E14 vg/monkey. The degeneration in the monkeys treated with AAV9/*AP4M1* correlated with a decrease in NCV (nerve conduction velocity) and response amplitude of the sural nerve (sensory) at the high dose, but

not peroneal nerve (motor), noted on days 45 and 77. Taken together, mild neuronal degeneration was noted in the lumbar DRG in both male and female at the highest dose of AAV9/*AP4M1*, but not AAV9/*MFSD8,* in WT rats and NHPs, but not in mice. These results, along with an absence of adverse clinical signs or mortality during the entire period of all studies, suggest that utilizing weaker (JeT) or moderate (UsP or JeT+Intron) promoters could be a mitigating strategy to reduce DRG toxicity under immunosuppression and both AAV9/*MFSD8* and AAV9/*AP4M1* at the dose of 1E15 vg/patient (which equals to 2.5E11 vg/mouse, 1.1E12 vg/rat, or 0.84E14 vg/monkey) had an acceptable safety profile for CLN7 or SPG50 patients. There are ongoing clinical trials in the US and/or Canada testing AAV9/*MFSD8* and AAV9/*AP4M1* at the dose of 1E15 vg/patient.

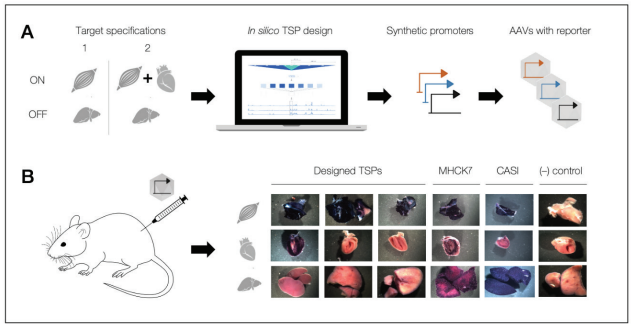

Figure 1: (A) Computational design of tissue-specific promoters (TSPs) for two target specifications. Each designed TSP was placed upstream from an alkaline phosphatase reporter gene and packaged into AAV vectors. (B) Testing three designed TSPs in vivo demonstrates that the observed expression profiles exhibit high dynamic-range between target and off-target tissue. MHCK7 shows expression in both target and off-target tissue. Four mice were dosed with each AAV payload and sacrificed after three weeks, at which point organs were harvested and stained for alkaline phosphatase activity.

AAV Vector Genome Biology and Engineering I

172 Machine-Guided Design of Tissue-Specific Promoters

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Adeno-associated viral (AAV) vector gene therapy has shown significant potential for treating otherwise intractable diseases, with three FDA-approved therapies since 2017. Most AAV therapeutic transgenes are expressed using ubiquitous promoters, such as the cytomegalovirus enhancer/chicken beta actin promoter, which are transcriptionally active in a broad set of tissues. This indiscriminate expression increases the risk of undesired off-target effects and tissue toxicity. To address these challenges, we have developed a computational method that integrates multi-omics analysis and machine learning to design tissue-specific promoters (TSPs) for targeted expression of AAV payloads. To validate our approach, we designed three TSPs to preferentially express in heart and/or skeletal muscle with minimal expression in liver (Figure 1A). The promoters were predicted to achieve strong expression only in target tissues, while also being short enough to package into an AAV alongside a transgene. For controls, we included two promoters that are currently being used in clinical AAV therapies: CASI (ubiquitous) and MHCK7 (heart/muscle specific). AAV vectors were generated that harbored an alkaline phosphatase reporter gene driven by the TSPs, which were subsequently administered to healthy 6-week-old C57BL/6 mice at a dose of 3E11 viral genomes per animal. The mice were sacrificed after three weeks and the skeletal muscle, heart, and liver were harvested and stained for alkaline phosphatase expression. Across the three designed TSPs, we observed a >1000-fold dynamic range in alkaline phosphatase activity between target and off-target tissues (Figure 1B). Surprisingly, we observed noticeable off-target liver expression for MHCK7. These results demonstrate the power of computational methods to achieve more precise expression control for in vivo gene therapy.

173 A New cceAAV Vector without a Mutant ITR is Efficient for Reducing Subgenome Generation

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Self-complementary AAV vectors (scAAV) have an engineered AAV genome which fold back on itself to form a special double stranded DNA and facilitate fast and efficient transgene expression. The approved spinal muscular atrophy (SMA) drug, Zolgensma, is based on scAAV vector. However, scAAV vectors are not always in a double stranded DNA form since they utilize a mutant ITR (mITR) to facilitate packaging of a complementary-stranded DNA. Inefficient resolution at the mITR leads to the generation and packaging of monomeric AAV genomes and subgenomic particles. All these non-canonical particles result in reduced transgene expression efficiency and potential immunological problems. To avoid these issues, here we developed a new class of scAAV vector, namely, Covalently Closed-End double stranded AAV genomes (cceAAV). The production of cceAAV vector bypasses the resolution step of the mITR altogether. Instead of using a mutant ITR, a 56-bp recognition sequence of protelomerase (TelN) were used to covalently link the top strand and the bottom strand and the vector can be produced with only a single ITR. To manufacture cceAAV vectors, cceAAV vector plasmid DNA was first digested with TelN enzyme and followed by a typical triple transfection protocol and standard AAV vector purification procedures. The yield of cceAAV vector has a comparative yield to scAAV vector. Denatured gel analysis of vector genomes as well as Charge Detection Mass Spectrometry (CDMS) analysis showed that cceAAV vectors produced generally has a more homogenous composition and decreased non-canonical particles as compared to traditional scAAV vectors. DNA bioanalyzer analysis shows that the genome integrity of this new cceAAV vector was significantly improved as well. Furthermore, cceAAV vector were able to avoid partial genome generation which was usually presented

in scAAV vectors. In animal studies, cceAAV vector with a factor IX expression cassette showed an increased 2~5 folds transgene expression over scAAV vectors. In summary, cceAAV vectors are the next generation of scAAV vectors which may be further developed for human gene therapy.

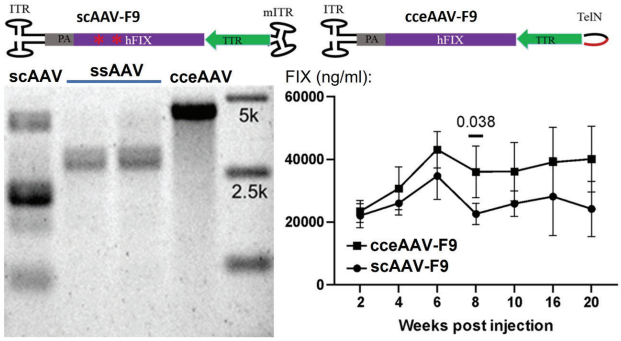

Figure: Top, Illustration of scAAV (self complementary AAV) and cceAAV. The difference is that cceAAV does not use a mutant ITR for making the complimentary genome. Left, DNA genome size distribution of AAV genomes from scAAV, ssAAV and cceAAV. Vector genomes extracted from scAAV, ssAAV and cceAAV are separated by denaturing gel and stained by cyber gold. It shows that cceAAV has a more homogenous genomes as comparing to scAAV vectors. Right, in vivo performance of cceAAV vector. Both scAAV and cceAAV vectors carrying the same factor IX expression cassette are injected into hemophilia B mice at the same dose of 1e11vg per mouse. cceAAV showed a 2-4 folds better expression than scAAV vectors.

174 Characterization of Promoter and Intron Interactions Affecting Transgene Expression in AAV Cassettes

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Recombinant Adeno-associated viral (AAV) vectors have tremendous potential for gene therapy. The AAV expression cassette contains several DNA elements that control the expression of the therapeutic transgene, which typically include a promoter, an intron, and a transcription terminator/polyadenylation signal. With the large number of promoters and introns that have been tested in preclinical and clinical studies, the combinatorial sum of possible cassette architectures is substantial. In many cases, these individual elements are put together in a modular manner, ignoring the possibility that interactions may affect functional outcomes. For example, we and others have shown that translation of alternative reading frames (ARFs) in AAV gene expression cassettes can result in unwanted peptides (ASGCT 2022). These ARF peptides can elicit immune responses and have been demonstrated to decrease expression of the desired therapeutic protein. Upstream ARFs can result from cryptic start codons in 5' untranslated regions, or from retained intron sequences due to splicing inaccuracies. To investigate these outcomes, we systematically evaluated cassette architectures for splicing accuracy, transgene expression, and ARF production. We constructed a set of AAV cassettes that contained three promoters and ten introns that drive expression of a reporter gene. We performed Ribo-Seq on cells that were transfected with these constructs. Next generation sequencing (NGS) libraries were sequenced on an Illumina NextSeq 1000, and ribosome protected fragment (RPF) reads were mapped to the plasmid sequence. Protein products were analyzed by western blot (WB) using an antibody against the reporter. In parallel, we analyzed splicing patterns by RT-PCR. We observed that expression levels of the reporter gene by WB were markedly different from constructs with different introns, even with the same promoter. Low expression from some introns was attributed to inefficient splicing as measured by splice PCR. Importantly, we detected the presence of upstream ARF peptides from multiple intron constructs. Reporter gene expression was substantially diminished from constructs that expressed upstream ARF peptides. Furthermore, we noticed a marked difference in splicing efficiency of the same intron in the context of a different promoter. These data collectively demonstrate that there are complex interactions between promoters, introns and transgenes that remain to be understood and that a universal plug and play system for expression cassette design may not always be optimal. Selection of introns is particularly important, and we believe plays a vital part in determining transgene expression and avoiding potential immunogenic ARF peptides. The methods we describe here, including Ribo-Seq, splice PCR and reporter gene assays, are valuable analytical tools and may be used to optimize performance of therapeutic cassettes.

175 Improving Codon Optimization for Gene Therapy Vectors Using Deep Learning

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Gene therapy seeks to introduce, modify, or manipulate the levels of genes in a patient to alter the biological properties of host cells for therapeutic purpose. One way to improve the efficiency of protein-coding transgene expression is by codon optimization. Current approaches for codon optimization, such as using a codon usage table or a biological index, are centered on eukaryotic versus prokaryotic usage rules, or are generalized to enhance mammalian expression. Differences among species, transcript abundances, protein length and structure, and differential expression of aminotransferases and transfer RNAs (tRNAs) between tissues or cell types are typically not considered in most codon optimization algorithms. These unmet considerations highlight the need for further research in the area, and are an unexplored facet for gene therapy vector engineering. It has been hypothesized that there are tissue-related synonymous codon biases that may allow sequence optimization to be effective in one tissue type but not in another. We have developed a novel recurrent-neural-network (RNN)-based codon optimization tool for cell type-specific codon optimization. We compiled a dataset of highly expressed genes in mouse myocytes (708), neurons (651), and hepatocytes (733) and extracted their corresponding codons and trained a deep neural network to predict the optimal codon usage patterns for the three independent cell types. Through the RNN architecture, we found that the sequential context of codons in highly expressed genes drove codon selection that was more similar to those observed in a set of 120 validation genes (40 genes from each cell type). We evaluated the outcomes of the codon-optimized sequences for each cell type by the distribution of codon frequencies, CpG dinucleotide content, GC content, and codon adaptation index (CAI), which compares the frequency of synonymous codons between the optimized genes

and those appearing within the reference set of highly expressed genes. We then used our model to optimize the sequences for two standard reporter genes, firefly luciferase (*FLuc*) and green fluorescent protein (*GFP*) for expression in neurons, hepatocytes, and myocytes. The codon-optimized constructs were tested in three mouse cell lines representing neuroblasts (Neuro-2a), myoblasts (C2C12), and hepatocytes (AML12). Expression of the codon-optimized *FLuc* transgene for each specific tissue type was enhanced in the correct corresponding cell line when compared to the non-optimized wildtype counterpart as evaluated by luciferase activity assays and Western blots. In addition, expression was superior to those conferred by codon-optimized transgenes obtained by a popular and publicly available algorithm. An unintended, but welcomed, consequence of our Deep Learning (DL)-driven codon-optimization trials was the reduction of CpG dinucleotides in the output sequences. Therefore, codon optimization using our algorithm does not directly counteract efforts to reduce or deplete CpG dinucleotides in vector designs. Current efforts to optimize therapeutically-relevant transgenes and evaluation with AAV vectors in mice are underway. Our novel approach was able to achieve improved protein expression compared to non-optimized and codon-optimized sequences produced by commercially available tools. Importantly, our DL approach confirmed that synonymous codon biases exhibit tissue specificity. DL-driven codon-optimization has the potential to drastically improve the efficiency of gene addition or gene replacement therapies for tissue-restricted diseases.

176 Searching the Hairpin in the Haystack: Tracing the Impact of AAV-ITR Mutations

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In Adeno-associated viral (AAV) vectors, engineering of the ITR (inverted terminal repeat, i.e., replication and packaging cis element) sequences may enhance vector productivity, safety and efficacy. Strikingly, however, the impact of the ITR sequence and structure on these parameters remains largely unexplored. To facilitate manipulation of the ITRs, we developed a toolbox for AAV ITR engineering which includes a vector construct design that allows easy modification of plasmid ITR sequences. This is complemented by a strategy for plasmid ITR sequence confirmation that can be used with conventional Sanger sequencing chemistry. To trace the ITR mutants during production and transduction, each ITR variant is associated with a specific DNA barcode in the 5‘ UTR of a transgene cassette on the virus genome. Titers of the resulting vectors based on this alternative design are similar to the traditional vector backbone. The integrity of the produced viral genomes and the presence of ITR mutants was examined by Nanopore sequencing. There, we discovered a previously undescribed and biologically highly interesting trans-acting ITR-repair mechanism in which ITR variants derived from different plasmids can serve as repair template. Consequently, by avoiding the presence of different ITR variants during vector production, this ITR repair mechanism can be circumvented, permitting AAV vector generation with mutant ITRs that are maintained in the virus genome. Subsequent barcode interrogation by deep sequencing enables the determination of barcode distribution at high resolution, which, in turn, allows to quantify the impact of the mutations on AAV genome replication and packaging. Barcode sequencing in extracted RNA can also serve as a qualitative and quantitative measure for effects of the ITR on vector transduction efficiency, despite the potential loss of the ITR by recombination in the nucleus. Altogether, the novel pipeline for ITR modification and tracing reported here forms the basis for the comprehensive analysis of alternative ITR designs and their function during AAV production and transduction, which should ultimately benefit the creation and optimization of next-generation AAV vectors not only on the capsid but also on the genome level.

177 Engineering mRNA Stability with Flaviviral Genomic Elements to Improve AAV Transduction

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Current AAV-based gene therapies require high vector doses, which may result in undesirable clinical side effects. Several constituents of the recombinant AAV genome such as promoter elements, introns, short DNA hairpins, and other regulatory elements have been engineered to enhance and control translational output. However, diversity and functional significance of post-transcriptional regulatory elements have yet to be investigated. Notably, strategies to increase nuclear mRNA export, improve rate of viral transcription, or significantly extend mRNA half-life, with respect to AAV transcript biology, are of particular interest. Here, we engineer AAV vector genomes containing sub-genomic flaviviral RNA (sfRNA) elements that boost transduction efficiency. Specifically, we appended sfRNA elements from Dengue virus serotype 2 (DENV2), Japanese Encephalitis virus (JEV), Murray Valley Encephalitis virus (MVEV), West Nile virus serotype, (WNV2), Yellow Fever virus (YFV), and Zika virus (ZIKV) to the 5’ and 3’UTRs of an AAV vector genome encoding firefly luciferase. Earlier results indicated that sfRNA elements from all tested flaviviruses dramatically increase transduction efficiency *in vitro* by extending mRNA half-life, with DENV2 sfRNA located in the 3’UTR exerting the greatest effect. We further investigate the DENV2 sfRNA and discover that only two dumbbell structures (DBs) were necessary and sufficient to increase AAV transgene expression *in vitro*. We then evaluated AAV9 vectors packaging a firefly luciferase cassette with DENV2 sfRNA, DENV2 DBs, or a control WPRE in the 3’UTR followed by intravenous dosing in mice. *In vivo* results corroborate the ability of flaviviral sfRNA elements to improve transgene expression compared to WPRE in a tissue specific manner, suggesting a potential for lowering vector doses. Engineered sfRNA elements hold promise for improving AAV gene expression, can potentially be substituted for traditionally used synthetic poly-A tails, and warrant further testing in larger animal models and human tissues.

178 Long Read Sequencing of rAAV Vectors Illuminates Origins of Contaminating Genomic Species

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AAV has become an increasingly important vector for gene therapy, with three approved products in the United States and over a hundred ongoing clinical trials. An extensive understanding of the composition of packaged genomes is critical to ensure the safety of AAV-based gene therapy products. While heterogeneity in the form of short, non-unit length genomes in packaged AAV genomic species has been known for decades, it has traditionally been assessed by gel-based methods that quantify size but not full sequence composition. Recent advances in sequencing technology, namely single-molecule long-read sequencing, have resulted in unprecedented insight into AAV replication and packaging. In this work, we used single-molecule long-read sequencing to examine self-complementary vector payloads containing varying regions of complex and homologous sequences. These vectors were produced from two commercial vendors using triple transfection in HEK293 cells and purified by ultracentrifugation. Our bioinformatic analysis identified two novel classes of contaminating genomic species. In our first observation, we found that repetitive elements with strong secondary structures can compromise genomic integrity by facilitating a loss of DNA between the regions of homology, possibly through slipped-strand mispairing during genome replication. This is distinct from previously described 'snap-back' genomes, which were also observed. The second observation contained plasmid backbone sequence in a configuration that cannot be accounted for by previously described 'reverse packaging' mechanisms. This genomic species originated from the nickable ITR, contained a small portion of payload, and had a chimeric sequence that joined to the plasmid backbone. These genomes were frequently packaged as concatemers linked by unnicked double-D ITRs. To our knowledge, the mechanism of reverse-packaging leading to these species is undescribed in the literature and would not be prevented by current vector engineering strategies such as enlarging the plasmid backbone or adding hairpins outside the ITRs. Work is currently underway to understand if these same species are also found in single-stranded rAAV vector preps. Our work presented here demonstrates the value of implementing a long-read sequencing pipeline for rAAV characterization, illustrated by the identification of novel genomic contaminants. While more work is needed to confirm the mechanism by which these genomic species arise, these observations suggest new strategies to maintain homogeneity and safety of rAAV preps.

Pharmacology/Toxicology Studies: Methodology

179 Assessing *In Vivo* Recombinant AAV DNA by Long-Read Sequencing after Gene Therapy

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Multiple *in vivo* studies have demonstrated reductions in long-term transgene expression as a potential drawback for recombinant adeno-associated virus (rAAV)-delivered gene therapies. While long-term reductions in transgene expression most likely arise from multiple factors, such as direct regulation of gene expression and vector loss due to cell turnover, it is becoming increasingly clear that potential heterogeneity in the sequence and structural integrity of rAAV DNA may also play a significant role. Fully understanding the underlying biology and potential heterogeneity of rAAV DNA requires better methods for interrogating both episomal and integrated rAAV DNA. Here, we optimized an assay harnessing the power of long-read sequencing and applied this approach to tissues from multiple nonhuman primates (NHPs) treated with rAAV vectors. Utilizing a hybridization-based approach with biotinylated probes tiled against the primary units of an rAAV genome (inverted terminal repeats [ITRs], promoters, transgenes, polyA signals, etc.), we specifically enriched for rAAV DNA in a way that minimizes the biases introduced by other approaches. This resulted in a pool of sequences directly representative of individual rAAV DNA molecules present in the samples. Using this approach and a custom bioinformatic pipeline, we assayed liver tissue samples from six NHPs collected approximately 2 years after dosing with rAAV vector to directly investigate *in vivo* rAAV sequences. These NHPs received vectors expressing a non-self transgene (enhanced green fluorescent protein, n=2) and a rhesus (n=2) or human (n=2) version of a transgene (low-density lipoprotein receptor). We identified differing amounts of rAAV reads from the samples based on the administered transgene, following the same trends observed via traditional assays such as genome copy number determined by quantitative PCR (qPCR) and consistent with transgene immunogenicity. In addition, the large majority (>90%) of the rAAV reads were identified as non-integrated, implying that the majority of AAV DNA at this ~2-year timepoint was maintained episomally. As the average high-fidelity read length in this study was ~6 kb, this approach enables a deeper investigation of the rAAV structure that is not easily provided by other techniques. In these samples, the large majority of rAAV DNA had undergone a dramatic rearrangement, with less than 40% of overall enriched rAAV reads across all samples containing an intact transgene. Very few reads show the expected pattern of concatemer formation (head-to-head or head-to-tail). Instead, different sections of the rAAV genome repeat multiple times in no discernable order, an observation that is seen across all samples and represents the most common outcome when individual long-reads are examined at the single-nucleotide level. Importantly, the samples with the highest identified transgene expression upon reverse transcription qPCR also had the most reads containing a functional transgene sequence, supporting the relationship between loss of transgene expression and potential rAAV DNA rearrangements *in vivo*. Together, the use of this hybridization and

long-read sequencing approach has broad applications for elucidating rAAV vector heterogeneity, including the finding that much of the *in vivo* rAAV DNA identified lacked the factors necessary to allow successful transgene expression. The generation of large unbiased data that is not dependent on only a few specific sequences, such as only the ITRs or transgene, as this assay allows, could help answer many questions pertaining to our overall understanding of rAAV-mediated gene therapy.

180 Retrieval and Quantification of Vector Integration Sites by Sonication Linker Mediated-PCR (SLiM-PCR): Efficiency and Applications

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Specialized PCR-based techniques combined with next generation sequencing (NGS) and bioinformatics analyses allow the retrieval and mapping of vector integration sites (IS) in the genome of transduced cells. Because IS are stable genetic marks, distinctive for each independently transduced cell and its progeny, their characterization in gene therapy (GT) applications allows to track transduced cells in different tissues or cell lineages over time, evaluate the clonal composition of the engrafted population, identify the targeted genes and quantify the relative abundance of clones harboring a specific IS to detect or exclude sustained clonal expansions. Here, we present our method for IS retrieval, Sonication Linker mediated- PCR (SLiM-PCR), in which vector/genome junctions are specifically PCR amplified from sheared genomic DNA and sequenced by NGS. The identification and quantification of IS is realized by dedicated software (VISPA2) while the analyses are performed by ISAnalytics, an R tool for comprehensive and integrated IS analysis. The abundance of each IS is obtained by counting the number of DNA fragments containing the same IS but variable in size depending on the shear site position. Hence, the number of shear sites assigned to an IS will be proportional to the initial number of contributing cells, avoiding the biases introduced by PCR amplification. We validated SLiM-PCR for quantitative LV and γ-RV IS retrieval, by setting up an experimental framework in which the genomic DNA of monoclonal samples harboring one LV or γ-RV vector IS in known genomic positions were mixed at different ratios (70-0%) with the genomic DNA of samples with IS randomly distributed in the genome. By applying SLiM-PCR on the different DNA mixes, we calculated the relative abundance of the clones with known IS versus the expected value, that was measured by digital PCR. SLiM-PCR showed a high correlation between the number of retrieved and expected IS ($R2 \simeq 0.9$). Indeed, the observed values of monoclonal samples' IS matched the expected (observed/expected = 0.96 ± 0.1) for all the dilutions up to the detection limit of 0.16% for LV and 0.1% for g-RV genomes over the total. Beside the experimental part, we have implemented Standard Operating Procedures and automated the whole SLiM-PCR process in 96/384-well plates with liquid handlers able to process hundreds of DNA samples per week. A laboratory information management system supports tracking the process and records all the relevant samples metadata used in the analytical bioinformatics process. In the last 2 years, our approach was successfully applied on DNA samples transduced by different vector types like gamma-retroviruses, LV, Sleeping Beauty Transposons and adeno-associated viruses. More than 4500 independent samples coming from 7 different GT clinical trials (97 patients) and more than 30 research and preclinical studies have been processed, generating a dataset of millions of IS. In conclusions, our results showed that SLiM-PCR is a reproducible, accurate and versatile method that combined with state-of-the-art infrastructures, automated laboratory procedures and novel bioinformatics and statistical tools allows to perform fast and high-throughput IS analyses.

182 Cell Isolation Following Apheresis Using an Immunomagnetic Beads Approach in Non Human Primates

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We evaluated a method used in a pre-clinical setting for hematopoietic replacement therapies and in the future, to produce cell therapies including CAR T for allogenic applications. Clinically, apheresis can be used to collect a blood component from an autologous donor through one of two methods: continuous mononuclear collection (CMNC) method, which employs outflow from one venipuncture and return flow through a second site or a dual lumen catheter, or the peripheral blood mononuclear cell (MNC) method which has a smaller dead volume and only a single venipuncture. In macaques, Neupogen (G-CSF, 50 µg/kg/day; administered on days 1 to 5) and Mozobil (Plerixafor, 1.0 mg/kg/day; administered on day 5, three hours prior to apheresis) were used to mobilize CD34+ hematopoietic stem cells from the bone marrow compartment. This 5-day process increased the CD34+ cell concentration in peripheral whole blood ranging from 1.8 to 15.5 x 10^3 cells/mL, up to values between 55.7 to 200.9 x 10^3 cells/mL prior to apheresis. CD11b-CD45+CD34+ cell concentration measurements were performed by flow cytometry at prior to mobilization, prior to Mozobil administration on Day 4 and 3 hours post Mozobil administration. Apheresis was performed using the Spectra Optia Apheresis System with outflow from the catheterized femoral or saphenous vein and return flow through the catheterized carotid or tibial artery. The system was initially primed with whole blood and then a CMNC collection was performed to harvest peripheral white blood cells, and adjusted to collect the buffy coat fraction rich in CD34+ cells. The subject can undergo the procedure for up to 6 hours, during which ionized calcium levels (targeted level of 1.0 to 1.3 mmol/L) were closely monitored and calcium gluconate was administered as needed to maintain safe ionized calcium levels in order to prevent citrate toxicity. In addition to the apheresis product bag, several microsamples for flow cytometry analysis were collected during the apheresis procedure to assess the effect of sample density (based on the collection color),

collection rate and time from Mozobil administration. Following the completion of the apheresis procedure, the apheresis product was subjected to a ficoll density gradient separation to isolate mononuclear cells. The mononuclear cell fraction was then immunomagnetically depleted of CD11b+ cells and the CD11b- fraction was once more stained, this time with anti-CD34 immunomagnetic beads to allow positive selection of CD34+ cells. Our methods have produced 73 to 95% purity of CD34+ cells across our studies, with a cell yield between 1.34 to 53.4 x 10^6 CD34+ cells across studies. Preliminary analysis of samples collected during the apheresis procedures suggested that while total cell counts were comparable across collection conditions, there were higher concentrations of CD45+CD34+ cells in samples collected within 5-6.20hrs post-mozobil administration, than later collections (up to 7.30hrs post-mozobil administration). In addition, when collected during the earlier timeframe, a higher concentration of CD45+CD34+ cells was obtained in a sample with a collection rate of 0.5mL/min, as compared to 1mL/min. Finally, the collection color (used as an index of cells density during collection), howed a trend to greater CD45+CD34+ concentration in samples collected in the following order: dark and middle then pale red patterns. We demonstrate high degrees of purity (>70%) in isolating CD34+ cells from apheresis product of non-human primates by immunomagnetic bead separation. This method can be harnessed to produce a large variety of cells for immunotherapies, as well as to develop, test, and bring to market CAR T cells produced though allogenic methods.

183 Characterization of AAV Integrations and Rearrangements from Long and Short Reads with RAAVioli

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Recombinant Adeno Associated Viral (rAAV)-based gene therapy (GT) applications have been successfully exploited for the treatment of several disorders. rAAV mainly remains episomal in the nucleus of transduced cells, however, numerous studies demonstrated integration of fragmented or full-length AAV DNA within the transduced cell genome where double-strand DNA breaks (DSBs) or nicks have occurred. Yet, preclinical studies revealed the occurrence of hepatocellular carcinoma and clonal expansion events consequent to rAAV insertions, posing safety concerns for their clinical use. However, bioinformatics tools able to identify AAV integration sites (IS) and characterize vector rearrangements are still missing. Here, we collected data from a humanized liver mouse model, where human primary hepatocytes have been transduced ex-vivo or in-vivo with a tomato expressing AAV. PCR amplicons or DNA fragments containing AAV vector portions were sequenced by both short paired-end and long reads and then analyzed by RAAVioli (Recombinant Adeno-Associated Viral IntegratiOn analysis), to characterize vector rearrangements and IS. Python and R scripts parse the alignments to identify IS and reconstruct rearrangements using CIGAR strings. We retrieved 811 and 370 IS from short paired-end Illumina reads and long PacBio reads respectively, confirming the higher efficiency of PCR-based approach in IS retrieval. The distribution of AAV IS was sparse in the human genome similarly in both datasets, and Albumin gene was the most targeted gene as expected. Furthermore, 32 ISs were in common between the two datasets, demonstrating the reliability of RAAVioli independently from sequencing platform adopted. Both datasets showed a similar percentage (~25%) of fragments with AAV rearrangements, however more than 2 rearrangements per fragment were retrieved only in long PacBio reads. Precision and accuracy of RAAVioli pipeline was assessed through simulated datasets obtaining scores >0.95 in IS identification and rearrangement characterization. These data demonstrated that RAAVioli is a comprehensive and flexible bioinformatic tool that can efficiently map AAV IS using long and short paired ends sequencing reads. These approaches are fundamental to characterize AAV integration and recombination events in gene therapy and gene editing applications, allowing and improving the assessment of safety in AAV studies.

184 CISC: A Multi-Purpose Enrichment Tool for HDR-Edited Human T-Cells Applicable for Treg and CAR-T Therapeutic Cell Products

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Engineered T cells represent an emerging therapeutic modality. However, complex engineering strategies can present a challenge for enriching and expanding therapeutic cells at clinical scale. Additionally, lack of in vivo cytokine support can lead to poor engraftment of transferred T cells, including regulatory T cells (Treg). We have established a cell-intrinsic selection system that leverages the dependency of primary T cells on IL-2 signaling. Novel FRB-IL2RB and FKBP-IL2RG fusion proteins, referred to as chemically inducible signaling complex (CISC), were identified permitting selective expansion of transduced primary CD4+ T cells in rapamycin supplemented media when delivered as a lentivirus. LV-transduced cells strongly activated the STAT-5 signaling pathway in response to CISC heterodimerization. The CISC was subsequently incorporated into HDR donor templates designed to drive expression of the Treg master regulator FOXP3 at the endogenous *FOXP3* locus or introduce a CD19-CAR and delete the endogenous T Cell receptor at the *TRAC* locus. Following FOXP3 editing of CD4+ T cells, CISC+ engineered Treg (CISC EngTreg) were selectively expanded using rapamycin and maintained Treg activity. Significantly, CISC EngTreg cells adoptively transferred into immune deficient mice showed increased engraftment and retention, and improved therapeutic efficacy in a xenogenic graft vs host disease model when mice were treated systemically with dimerizer. *TRAC* edited CD4+ T cells displayed efficient endogenous TCR knockout in combination with CD19-CAR expression, and functional CISC CAR-T Cells were selectively enriched in the presence

of rapamycin. In summary, CISC provides a robust platform for the in-vitro selection of multiple HDR edited therapeutic T Cell products and can promote in-vivo efficacy of engineered Treg cells.

185 Novel Tools for Gene and Cell Therapy Safety: Optical Long-Read Genomics for Detection and Characterization of On- and Off-Target Transgene Integrations and Off-Target Structural Variants

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During the development of gene and cell therapy products, especially the strategies involving a 'transgene' delivered to patients or cells, to repair a gene or rescue its function, integration of said transgene raises safety concerns. For gene repair via integration in gene therapy, or transgene knock-in approach in cell therapy, on-target, and any potential off-target transgene integration needs to be well characterized. For example, the genomic locations of such integrations, frequency, size (full length or not), copy number, and possibly orientation are a few of the parameters that need to be determined. AAV-based gene therapies, relying on the episomal expression of the delivered transgene need to be screened for possible unintended integration of such transgenes into the host genome. Additionally, genome-editing manipulations can generate other non-transgene-related structural variants (SVs) in the host genome, which can pose safety risks. In cell therapy candidates, the cellular expansion step can induce further genomic changes. Some commonly employed approaches for measuring these genetic changes either suffer from PCR biases and short read lengths (150-300bp) which miss capturing larger SVs (e.g. NGS-based targeted assays) or have too low resolution (e.g. karyotyping, FISH for chromosome-level changes). We are addressing these concerns by employing optical genome mapping (OGM) approach, which involves imaging and analyzing ultra-long (150kb-Mbp) single molecules of DNA, to detect SVs >0.5kb. It can capture the entire SV and long stretches of neighboring DNA in one read, thus presenting a complete picture of the genetic change. It is a PCR-free, target-agnostic approach and allows genome-wide mapping of the detected SVs. Using this approach we were able to accurately detect and characterize multiple variants of on-target integrations, their respective frequencies, size and copy number, in our studies. We also captured genome-wide off-target SVs, ranging from a few kb in size to chromosomal level, both post-genome-editing and during the cell expansion stage. Thus we demonstrate that this approach fills a gap in the safety evaluation and deeper characterization of gene and cell therapy candidates and can be a simple and efficient approach for such purposes.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases II

186 Phase 1/2 Clinical Trial of Autologous Hematopoietic Stem and Progenitor Cell Gene Therapy for Cystinosis

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Cystinosis is a lysosomal disorder characterized by cystine accumulation within the lysosomes of all organs caused by mutations in the *CTNS* gene encoding the transmembrane lysosomal cystine transporter, cystinosin. Major complications of cystinosis include early renal Fanconi syndrome, chronic kidney disease, renal failure, and ocular pathology that can lead to blindness. Cystinosis also affects the heart, thyroid, skeletal muscle, pancreas, and CNS, eventually causing premature death in early adulthood. Cysteamine delays, but does not stop, disease progression. Here we report results from the phase 1/2 open-label clinical trial (NCT03897361) evaluating safety and efficacy of CTNS-RD-04 in adult patients with cystinosis. CTNS-RD-04 consists of autologous $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) transduced with a lentiviral vector (LV) carrying the *CTNS* cDNA encoding for cystinosin (CCL-EFS-CTNS-WPRE). Peripheral blood $CD34^+$ HSPCs are collected via apheresis after mobilization with G-CSF and Plerixafor and transduced with CCL-EFS-CTNS-WPRE LV. Myeloablative-busulfan conditioning at a targeted AUC of 90 mg×h/L is followed by CTNS-RD-04 infusion. Oral and topical cysteamine are withdrawn prior to infusion. The trial is fully enrolled, and six participants (ages 20 to 46 years) have been treated with CTNS-RD-04 with follow-up ranging from 1 to 36 months. CTNS-RD-04 cell doses ranged from 3.63×10^6 to 9.59×10^6 $CD34^+$ cells/kg with VCNs ranging from 0.6 to 2.9 copies/dg. In all five infused patients with 42+ days of post-CTNS-RD-04 infusion follow-up, polyclonal hematopoietic reconstitution occurred. Peripheral blood VCN at 12 months post-gene therapy ranged between 0.43 to 1.99. In the five first treated patients, white blood cell cystine and tissue cystine crystals in skin and rectal mucosa decreased compared to Baseline. All subjects are no longer taking oral cysteamine. Patient 2 restarted eyedrop cysteamine one-year post-infusion. No adverse events related to drug product and no serious adverse events have been reported to date. Updated data will be presented.

187 Manipulating Macrophage Phenotype to Improve Inflammatory Bowel Disease Outcomes

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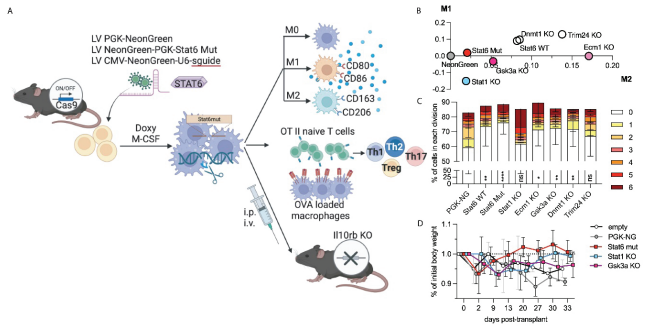
Inflammatory bowel disease (IBD) is a chronic inflammatory condition that affects the intestine. Environmental factors, infectious microbes, genetic susceptibility, and a dysregulated immune system can result in mucosal inflammation. The mechanisms behind establishing and maintaining intestinal tolerance are complex, involving anatomy, cells, and humoral factors. Intestinal macrophages (Mφ) play a key role in controlling gut immune responses and maintaining homeostasis. Substitution of Mφ with healthy ones improves IBD symptoms in a genetic mouse model of IBD (*Il10rb* deficiency). Depletion of endogenous Mφ was necessary to achieve therapeutic benefit. We surmised that this step was necessary to reset the Mφ niche and to prevent the polarization of incoming cells by the hyper-inflammatory environment. We hypothesized that locking Mφ before injection into an anti-inflammatory (M2) phenotype could improve therapeutic efficacy and eliminate the need for conditioning. In this study, we used CRISPR/Cas mediated knock-out (KO) of 12 genes or lentiviral addition of one gene to enforce an M2 phenotype in Mφ. Based on cell surface marker expression and cytokine secretion, we selected KO of *Stat1*, *Ecm1*, *Gsk3a*, *Dnmt1* and *Trim24*, or overexpression of constitutively active *Stat6* as promising candidates for promoting tolerance in IBD (A). All these candidates except *Stat1* KO led to an up to 3-fold upregulation of cell surface markers associated with M2 polarization. In contrast, KO of *Stat1* led to impaired acquisition of the inflammatory (M1) markers CD80 and CD86 in response to LPS and IFNγ (B). All candidates led to reduced expression of pro-inflammatory cytokines and chemokines upon LPS and IFNγ stimulation compared to control Mφ. Next, we evaluated the capability of genetically engineered Mφ to modulate naïve T-cell activation and polarization *in vitro*. For this, OVA-peptide stimulated macrophages were mixed with OT-II naive CD4+ T cells (C). *Stat1* KO did not change the proliferation of OVA-specific T cells compared to control Mφ but drastically increased the percentage of Gata3+ T cells, indicating increased differentiation towards Th2. While no significant changes were observed in T-reg induction, expression of mutant *Stat6* or KO of *Gsk3a* and *Dnmt1* led reduction of T cell proliferation. Finally, we evaluated the therapeutic potential of genetically engineered Mφ in *Il10rb*-deficient IBD mice. Transplantation of Mφ engineered to express mutated *Stat6* or KO of *Stat1* or *Gsk3a* led to improved clinical symptoms of IBD. We observed body weight gain (D), improved colon histopathology, and altered T cell activation in the mesenteric lymph nodes. This study highlights the potential of targeting Mφ polarization as a therapeutic strategy for IBD and possibly other inflammatory disorders, particularly those that are unresponsive to conventional anti-inflammatory therapy.



188 Impact of Genetic Diversity on Gene Therapy Efficacy

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Adeno-associated virus (AAV) vectors have been successfully applied in clinical trials in patients with many genetic disorders. Although promising results have been achieved, variable transgene expression and immune related adverse events remain a concern. Preclinical studies typically rely on inbred mouse disease models. The lack of genetic diversity in these inbred mice, in contrast to the human patient population, may limit the predictive ability of mouse models for therapeutic efficacy and immune responses in patients. To address this, we utilized the collaborative cross (CC) mouse strains to elucidate the role of genetic background on transgene expression and immune responses to AVV gene therapy. The CC is a panel of more than sixty recombinant inbred mouse strains derived from eight founder strains. These strains provide a reproducible source for genome-wide genetic variation for studies of human disease and drug development. For our study, we administered 3E11 particles of AAV8/GAA (acid α-glucosidase) vectors, *via* retro-orbital injection into 16 collaborative cross strains and the eight founder strains. This clinical vector, used in our Pompe program, directs liver expression of secreted GAA, which can be measured in serum by immunoblot or enzymatic activity. We monitored serum GAA activity over a six-month period. At month six, mice were euthanized, and we collected tissues to detect AAV genome copy numbers and to measure the phenotype and function of T cells in the spleen and antibody levels in serum. Overall, we observed a dramatic difference in transgene expression and immune response across the different CC and founder strains. Serum GAA activity varied by >100 fold across strains and displayed strain-specific expression kinetics. GAA expression in all strains increased in the first weeks and plateaued after week four. In distinct genetic strains, GAA expression

decreased over time. In some strains, higher GAA activity correlated with increased vector genomes in liver, suggesting differences in biodistribution and/or liver transduction were responsible. In other strains, however, there was discordance between transgene expression and liver transduction. Founder strains also differed in transgene expression and kinetics, to a lesser extant than the CC strains. Genetically diverse strains also demonstrated vast differences in the proportions of innate, B and T cell subsets in the spleen and varied in their reactivity to polyclonal T cell stimulation. AAV8 binding and neutralizing antibodies were detected in all animals, with some genetic strains having significantly higher anti-AAV antibody levels. In addition, one genetic strain demonstrated an AAV8 specific T cell response. GAA expression was inversely associated with the magnitude of anti-GAA antibodies in serum. A GAA specific T cell response was observed in two genetic strains, one of which had the lowest levels of GAA in serum. In summary, the data generated from this study shows that the genetic background of mouse strains has a substantial impact on gene therapy efficacy and immune response. This study argues for the use of diverse mouse strains on preclinical drug development. Since the parentage of CC strains is defined, it is possible to map phenotypic outcomes to genomic regions. More detailed knowledge about these influences may enable us to screen patients to ensure therapeutic efficacy, predict the immunological response and adjust the treatment to the individual patient.

189 Effective Readministration of AAV Gene Therapy Prevents Tumorigenesis in Infant PFIC3 Mice, a Mouse Model Prone to Hepatocellular Carcinoma

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Introduction: Treating pediatric patients with liver-targeted AAV-based gene therapy faces the significant obstacle of a loss of therapeutic effect over time. Indeed, natural liver growth and hepatocyte regeneration result in a decline in AAV episome-driven expression therefore necessitating readministration to restore AAV episome pools. Readministration is only feasible if steps are taken to prevent or overcome immunological memory which hampers AAV infection of target cells due to the presence of neutralizing antibodies. The development of a strategy for successful readministration may be essential for many pediatric liver disorders. For example, progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare hepatic condition that typically presents during infancy or early childhood and results from improper transport of phosphatidylcholine from hepatocytes into the bile due to mutations in the *ABCB4* gene. We have previously demonstrated successful therapeutic reversal of disease manifestations in a mouse model of PFIC3 with a single administration of VTX-803(8), a recombinant AAV8 carrying a codon optimized version of the human *ABCB4* gene. However, the long-term effects of VTX-803(8) treatment, including any effect on the development of hepatocellular carcinoma frequently observed in PFIC3 mice, had not been examined. **Methods:** We thus carried out further testing of VTX-803(8) in infant PFIC3 mice. Immunotolerogenic rapamycin-containing nanoparticles (ImmTOR) were co-administered with VTX-803(8) with the aim of inducing immune tolerance for AAV in very young mice (2 weeks old). Two weeks later VTX-803(8) was readministered to gauge immunological tolerance to a second AAV infection following a first administration admixed with ImmTOR. Long-term effects on PFIC3 disease manifestations and evidence of tumorigenesis were analyzed 8 months later. **Results:** Only animals given ImmTOR on the first administration and treated with a second VTX-803(8) administration achieved a long-term therapeutic effect as evidenced by normalization of serum biomarkers of cholestasis, hepatosplenomegaly, phospholipid content in the bile and liver fibrosis. At 8 months post-injection, control saline-treated animals and animals treated only one time with VTX-803(8) (either with or without ImmTOR) or two times without ImmTOR presented with liver lesions, gallstones (41%), and atypical hepatocellular neoplasms or dysplastic nodules detected via histopathological analysis (52%). In contrast, all mice that achieved successful transgene expression and reversal of cholestasis following repeat VTX-803(8) administration with ImmTOR did not show any sign of tumorigenesis. Further immunohistochemical and gene expression analyses of preneoplasia markers supported these findings, including differences in hepatocyte proliferation, bile duct proliferation and incidence of DNA double strand breaks. **Conclusions:** These outcomes support AAV-mediated gene therapy as a highly efficient and safe modality for a curative approach to inborn errors of metabolism including in very young patients. It also provides evidence countering the strong-held belief that AAV treatment is liable to increase the risk of hepatocellular carcinoma particularly when given to pediatrics or in patients with liver damage, which is an essential evaluation for the safety determination of this powerful therapy.

190 mRNA-3927 Therapy for Propionic Acidemia: Interim Data from a Phase 1/2 Study

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Background: Propionic acidemia (PA) is a rare and severe inherited metabolic disorder caused by pathogenic variants in the propionyl-coenzyme A carboxylase (PCC) α or β subunits (*PCCA* and *PCCB* genes, respectively) leading to PCC deficiency and subsequent accumulation of toxic metabolites. PA is characterized

by recurrent life-threatening metabolic decompensation events (MDEs) and multisystemic complications. Currently, there are no effective therapies for PA that target the underlying enzyme defects. mRNA-3927 is an investigational dual mRNA therapy that encodes for PCCA and PCCB that has shown to restore functional PCC activity in preclinical models. Results from an interim analysis of the first-in-human trial of mRNA-3927 are reported here. **Methods:** This ongoing global, phase 1/2, open-label, multicenter, dose-optimization trial (NCT04159103) evaluates the safety, tolerability, and pharmacology of mRNA-3927 in participants aged ≥1 year with genetically confirmed PA. The trial uses a staggered enrollment, dose-escalation approach to evaluate the intravenous administration of mRNA-3927. The initial dosing regimen was 0.3 mg/kg administered every 3 weeks (Q3W); subsequent doses were administered every 2 weeks (Q2W). Participants who complete the dose optimization trial (10 doses) are eligible to continue treatment in an open-label extension study (NCT05130437). Primary outcomes are safety and tolerability; secondary and exploratory outcomes include pharmacology and evaluation of potential plasma biomarkers and frequency and duration of MDEs, respectively. **Results:** As of January 5, 2023, 14 participants were enrolled across 5 dose cohorts: 0.3 mg/kg Q3W (n=4), 0.3 mg/kg Q2W (n=3), 0.45 mg/kg Q2W (n=3), 0.6 mg/kg Q2W (n=3), and 0.9 mg/kg (n=1). Nine participants completed the study and enrolled in the open-label extension (0.3 mg/kg Q3W [n=3], 0.3 mg/kg Q2W [n=3], 0.45 mg/kg Q2W [n=3]). A total of 214 doses were administered across both studies with a median treatment duration of 27.3 weeks (range, 3.0-85.6). No dose-limiting toxicities or study discontinuations due to drug-related treatment-emergent adverse events (TEAEs) have occurred. Across both studies, drug-related TEAEs were reported in 5 patients; all were grade 1 or 2. Preliminary analyses for patients administered mRNA-3927 suggest reductions in plasma biomarker levels. Additionally, in participants who reported MDEs in the 12 months prior to dosing, reductions in number and duration of MDEs were observed after the start of treatment with mRNA-3927. **Conclusion:** To date, mRNA-3927 has been well-tolerated at the doses administered, with encouraging early signs of dose-dependent pharmacology and potential clinical benefit.

191 A Pancreatic Gene Therapy Delivery Platform for the Treatment of Type 2 Diabetes

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The pancreas plays a key role in regulating metabolism and is central to the pathogenesis of diabetes. Local delivery of adeno-associated virus (AAV) may represent a strategy to durably modulate pancreatic function and improve the risk-benefit profile of gene therapy for type 2 diabetes (T2D). We developed a novel AAV-based gene therapy platform to enable local and durable production of therapeutic proteins by the pancreas. Our aim was to assess the efficacy of AAV-driven beta-cell (β-cell) restricted production of a glucagon-like peptide 1 receptor agonist (GLP1RA) in a murine T2D model and establish the feasibility of a novel, endoscopic, ultrasound-guided, automated delivery system and route of administration (ROA) to enable local pancreatic gene therapy delivery in a porcine model. A GLP1RA transgene was chosen for proof-of-concept (POC) efficacy analyses. In db/db mice, a standard T2D model, we showed durable and dose-dependent improvements in glycemic control after a single intraperitoneal injection of an optimized AAV-based GLP1RA vector with a β-cell-restricted promoter (AAV-GLP1RA; n=8 per group). A 70% reduction in fasting blood glucose ($p<0.0001$), a 1.9-fold increase in fasting insulin ($p<0.01$), and improvements in glucose tolerance were observed to 10 weeks post-injection compared to controls. GLP1RA protein was islet-restricted with an average of 4.6% of cells staining positive by immunohistochemistry. Serum levels of GLP1RA were undetectable, suggesting that durable improvements in glycemic control were due to local intra-islet GLP1RA production and signaling. To enable local AAV delivery, an endoscope integrated with a proprietary delivery system was used to optimize direct pancreatic injections in Yucatan pigs. Over 20 pigs (30-40kg) have been injected with AAV via our ROA; and parameters including serotype (AAV8, AAV9), promotor (CMV, β-cell restricted), and dose (5E12-1.5E14 VG, ~1E11-5E12 VG/kg) were evaluated. We observed dose-dependent transduction activity of the pancreas by AAV as determined by green florescent protein (GFP) expression 3-4 weeks post-injection. In high dose (1.5E14 VG, ~5E12 VG/kg) AAV9-infused animals (n=4), an average of 46.7% of pancreatic cells were transduced with some regions showing over 95% activity, suggesting that this ROA holds promise for high on-target transduction by AAV-based pancreatic therapies. To establish the on- and off-target safety for AAV via this ROA, we assessed parameters including serum levels of pancreatic enzymes, AAV biodistribution, and organ histopathology. A dose-dependent elevation in serum lipase (up to 2.5-fold above baseline) was observed in some AAV-CMV-GFP treated pigs, which resolved by 72 hours post-injection. Biodistribution data from multiple tissues support high AAV exposure to the pancreas with limited off-target transduction for most of the assessed tissues. Dorsal root ganglia (DRG) transduction was also noted across multiple doses, with a relatively low dose of scAAV9-CMV-GFP (5E13 VG, ~1.3E12 VG/kg) leading to hindlimb ataxia in one pig. Corresponding histopathology showed pancreatic and DRG inflammation, which was attributed to GFP reporter immunogenicity, as using a β-cell-restricted promoter eliminated these findings (n=7). In aggregate, these studies provide POC for the durable efficacy of pancreatic gene therapy and establish the feasibility of a novel delivery system and ROA to directly target the pancreas. Further transgene, serotype, promotor, delivery system, and safety studies are ongoing toward the advancement of our platform for the treatment of metabolic diseases, including T2D.

192 Rescue of Glutaric Aciduria Type I Mice by Liver Directed Therapies

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Glutaric Aciduria Type I (GA-1) is an inborn error of metabolism with a severe neurological phenotype caused by the deficiency of Glutaryl-CoA dehydrogenase (*GCDH*), a downstream enzyme of the lysine catabolism pathway. GA-1 is an autosomal recessive disorder that affects 1:100,000 live births and is more common in certain populations with a founder mutation such as the Lumbee, the Oji-Cree and the Amish communities. Lysine catabolism pathway disruption by GCDH deficiency causes neurodegeneration and serious neurological problems in patients with GA-1. Currently, the only treatment option available for patients with GA-1 is lysine restriction, carnitine supplementation and emergency care during acute crisis. Previous literature suggests that the toxic catabolites responsible for the neuropathological phenotype of GA-1 are produced locally in the brain. As dietary lysine is mainly catabolized by hepatocytes, we hypothesized that restoring lysine catabolism in the liver is sufficient to restore GA-1 phenotype and propose a liver-directed gene therapy approach. 4-6-week-old *Gcdh*$^{-/-}$ mice exposed to high protein diet die within 5 days showing signs of lethargy and failure to thrive. In a series of experiments using knockout mice and hepatocyte transplantation, we first demonstrated that GA-1 catabolites in the brain originate from the liver and can cross the blood brain barrier. Next, we performed liver directed therapies to restore hepatic lysine catabolism function. For the first therapeutic approach, we injected *Gcdh*$^{-/-}$ mice with an Adeno-Associated virus (AAV) expressing *Gcdh* gene under a liver specific promoter. Introducing *Gcdh* cDNA in GA-1 adult mice rescued them from high protein-induced lethality, while mice injected in the neonatal phase succumbed when exposed to the diet at 4 weeks of age, possibly because of hepatocyte proliferation and consequent dilution of the viral episomes. As a second therapeutic approach, we blocked the production of toxic catabolites by deleting hepatic Aminoadipate-Semialdehyde Synthase (*Aass*), the first enzyme in the lysine catabolic pathway, using CRISPR/Cas9 genome editing tools delivered by AAV vectors. Liver specific *Aass* deletion in neonates protected GA-1 mice from high protein exposure at 4 weeks of age. Both therapeutic approaches could also reverse the typical neuropathological phenotype and restore the motor performance of GA-1 mice. Our results show that the neurological and metabolic phenotype of GA-1 mice can be rescued by different liver-directed gene therapies. Our findings question current pathophysiological understanding of GA-1 and reveal for the first time a targeted therapy for this devastating disorder.

Novel Genetic Approaches for Muscle Diseases

193 CRISPR/Cas9 Homology-Independent Targeted Integration of Exons 1-19 Restores Full-Length Dystrophin in Mice

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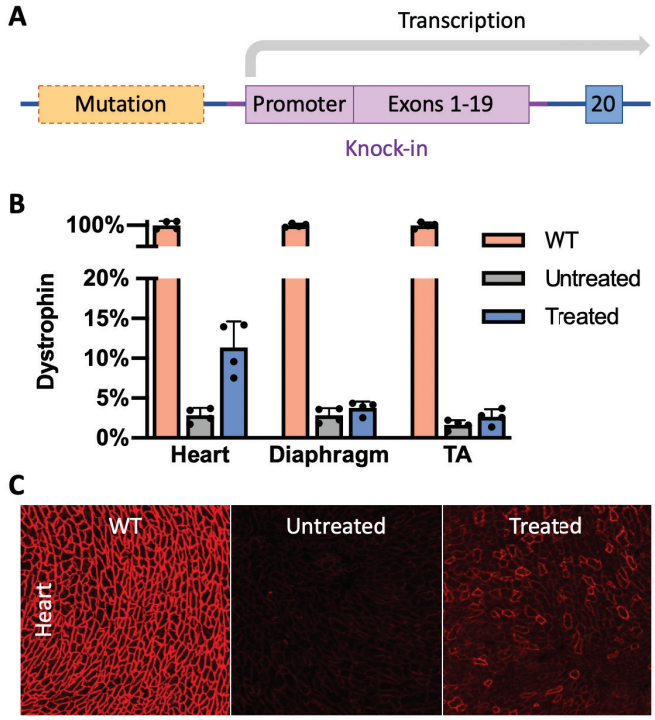
Background: Duchenne muscular dystrophy is an X-linked disorder typically caused by out-of-frame mutations in the *DMD* gene. Most of these mutations are deletions of one or more exons, which can theoretically be corrected to the wild-type sequence through CRISPR/Cas9-mediated knock-in of DNA fragments in *DMD*. Homology-independent targeted integration is a mechanism for achieving such a knock-in without reliance on homology-directed repair pathways, which are inactive in muscle. **Aims:** We designed a system based on insertion into intron 19 of a DNA fragment containing a pre-spliced mega-exon encoding *DMD* exons 1-19, along with the MHCK7 promoter (Figure A). In a series of experiments, we optimized the design of the system and demonstrated its ability to restore dystrophin expression in a mouse model of Duchenne muscular dystrophy. **Methods:** A pair of AAV vectors was used for systemic delivery of the gene editing system in neonate and adult mice carrying a *Dmd* exon 2 duplication. One vector encoded the guide RNA and knock-in construct, while the other encoded the *S. aureus* Cas9 enzyme. Biodistribution, knock-in efficiency, and transcript correction were measured by digital droplet PCR. Dystrophin expression was measured by quantitative immunofluorescence and capillary western immunoassay. Next-generation sequencing was used to assess editing outcomes at the target site and to screen for off-target activity. **Results:** Optimization experiments demonstrated that a Cas9:donor AAV ratio of 1:5 was more effective than a 1:1 ratio. Meaningful gene editing efficiency could be achieved with Cas9 under the control of the CMV, MHCK7, or SPc5-12 promoters, with a trend towards higher efficiency with SPc5-12. The CK8 promoter was ineffective. In all experiments, efficiency was higher in the heart than in skeletal muscles. Following optimizations, this system achieved editing of 1.4% of genomes in the heart, leading to 30% correction at the transcript level (measured as a fraction of all *Dmd* transcripts) and restoration of 11% of normal dystrophin levels (Figure B), as well as an increase in percent dystrophin positive fibers (Figure C). These data provide proof-of-concept for a gene editing system that could restore full-length dystrophin in individuals carrying mutations upstream of intron 19, accounting for approximately 25% of Duchenne muscular dystrophy patients.



194 Orthogonal Long- and Short-Read Sequencing after AAV-CRISPR Reveals On-Target Heterogeneity and Chimeric AAV-*Dmd* Transcripts

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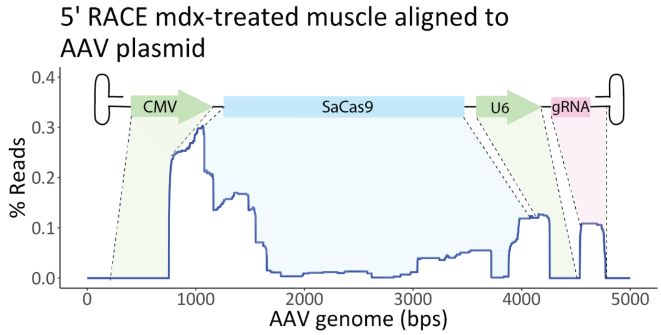
Adeno-associated virus (AAV)-mediated delivery of CRISPR components has yielded promising preclinical results for treating Duchenne muscular dystrophy (DMD). However, the post-transduction consequences of AAV-CRISPR, including AAV integration, require further characterization to develop a comprehensive screening method. The common method for screening CRISPR-edited outcomes involves PCR-enriched short-read sequencing at cut sites. However, PCR bias produces unreliable quantification of on-target edits. Here, we analyze orthogonal long- and short-read sequencing approaches to determine the comprehensive outcome of CRISPR technologies at the DNA and RNA-level in DMD. These approaches have been replicated with *in vitro* mouse myoblast AAV and electroporation edited samples to determine editing outcome differences *in vitro* and *in vivo*. We performed intramuscular injections of AAV-CRISPR or liposomes containing SpCas9 mRNA excising exon 23 using dual gRNAs at intron 22 and 23, in *mdx* and wild-type mice. PCR-amplified short-read sequencing revealed indels at both cut sites. While tagging with unique molecular identifiers (UMIs) can ameliorate some PCR bias, this solution fails to detect large structural variants or the breadth of AAV-integration events. In comparison, Nanopore-sequencing results of a 10kb PCR-enriched amplicon at the target site produces a broader catalog of editing events but similarly excludes quantification of large structural on- and off-target modifications. To reduce bias, we developed short- and long-read sequencing libraries for PCR-enriched samples with UMI's to compare with nanopore Cas9-targeted sequencing (nCATS) samples of similar lengths. Using nCATS, we were able to successfully detect structural modifications and large integrations. To compare with a previously identified quantification standard, we developed a Tn5 tagmentation library loaded with UMI's and internally PCR-amplified within cut sites to quantify on-target deletion percentages. We also examined the transcriptional impact of AAV-CRISPR on *Dmd* in *mdx* and wild-type mice. Nanopore-sequencing of 3kb PCR-enriched cDNA libraries failed to provide sequencing depths necessary to detect editing events. However, 5' RACE nanopore libraries revealed the presence of AAV integration at the RNA-level ranging up to 0.3% of reads creating a Cas9-dystrophin chimeric RNA with the transcriptional start site originating downstream of the internal CMV promoter in the AAV genome. To further examine AAV-integration profiles in the genome, we performed 3' RACE using the integrated AAV genome as the gene-specific primer to detect alternative off-target locations of AAV integration and additional chimeric transcripts. Owing to transcriptional regulation differences between *mdx* and wild-type mice, investigating the outcomes of AAV-CRISPR in both lines produced differing results. We also used next-generation enrichment approaches to investigate prime editing-mediated exon skipping or targeted integration by PASTE for DMD *in vitro*. Our results indicate that double-stranded break-free methods are more precise but have a low efficiency at target sites. Our results contribute significant considerations for the translation of AAV-CRISPR for DMD or similar monogenic diseases and facilitates the redefinition of sequencing standards for CRISPR readout.



195 AAV-NoSTOP Gene Therapy for Nonsense Mutation Mediated Dysferlinopathy

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Dysferlinopathy is a group of heterogenous autosomal recessive muscular dystrophies caused by loss-of-function mutations in the *DYSF* gene, which encodes dysferlin, a 237-kDa transmembrane protein involved in membrane repair. Currently, there is no treatment available for dysferlinopathy. Our lab previously reported AAV-NoSTOP (Wang J. et al. *Nature* 2022), an *in vivo* gene therapy approach that uses AAV-delivered suppressor tRNA (sup-tRNA) to promote readthrough of a pathogenic premature termination codon (PTC) causing mucopolysaccharidosis type I (MPS-I). One advantage of

AAV-NoSTOP is that it obviates the need to deliver a cDNA that may exceed the packaging limit of AAV vectors, such as the 6.2 kb *DYSF* cDNA. Intriguingly, a sup-tRNA may be broadly applicable to target the same PTC (e.g., UAG) underlying various diseases. Here, we generated a knock-in mouse model of dysferlinopathy carrying a *Dysf* nonsense mutation, and tested the therapeutic efficacy of the same sup-tRNA that we originally developed in the context of MPS-I. Using CRISPR-based genome editing, we generated mice harboring the *Dysf* p.Y522* mutation (TAT to TAG) that is commonly found in Korean patient population. In homozygous mice ($Dysf^{KI/KI}$), dysferlin protein in the heart and skeletal muscle was undetectable by Western blot, and *Dysf* mRNA was significantly reduced due to nonsense-mediated mRNA decay (NMD). To target muscle, we packaged the UAG-targeting, tyrosine tRNA-derived sup-tRNA gene (sup-$tRNA^{Tyr}$) into AAVMYO, a muscle-tropic capsid. In a pilot experiment, we treated adult $Dysf^{KI/KI}$ mice with AAVMYO.sup-$tRNA^{Tyr}$ via systemic tail vein injection, and found that dysferlin protein was restored to 15% of wild-type level in the heart, although only 2% restoration was observed in skeletal muscles. These preliminary results suggest that the $Dysf^{KI/KI}$ mouse model is suitable to test AAV-NoSTOP, and that the sup-$tRNA^{Tyr}$ is potentially efficacious to target UAG PTCs in various gene and disease settings. It was recently shown that 10-20% of normal dysferlin expression in mice is sufficient to prevent the disease phenotype. To further increase PTC readthrough and dysferlin restoration in the skeletal muscle, we are testing more potent muscle-tropic capsids, optimizing the transgene design, and exploring other sup-tRNAs.

196 Development of Myorganoids for Therapeutic Evaluation of Gene Therapy Products in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a rare genetic disease that affects the skeletal and cardiac muscles, leading to progressive muscle wasting and premature death. DMD is caused by the lack of Dystrophin, a muscle structural protein required for the biomechanical support of myofibers. Despite the enormous progression done in the gene therapy field using a short form of dystrophin (micro-dystrophin), there is still no cure for the disease. Moreover, the immunogenic risk associated to the high dose of AAV makes necessary the identification of other therapeutic options beyond or in support of gene therapy. This clearly indicates that animal models, although particularly important for pilot studies, do not recapitulate in full human DMD pathology and cannot predict the real efficiency and safety of a treatment, thereby highlighting the urgent need to develop *in vitro* model mimicking human DMD phenotype suitable as screening platform for test therapeutic products. The generation of reliable DMD human models in a dish would also limit animal wasting as it would allow a preselection of therapeutic candidates for further in vivo investigation (toxicology, immunological studies). Among the *in vitro* cellular models, organoid-like structures are becoming an appealing resource for disease modeling and replacement of animal models. As such, the use of 3D cultures and biomaterials allows the reconstitution of tissue architecture and microenvironment that are instrumental for pathophysiological studies Here, we describe the generation of MYOrganoids based on the optimization of a previously reported protocol that allows rapid generation of a pure population of myogenic precursors cells. MYOrganoids were generated by 3D culture of patient-specific iPSC-derived muscle precursors and isogenic fibroblasts within a collagen-based scaffold and silicone stretchers which supports the growth of ring-shaped tissues under mechanical load. MYOrganoids showed structural and functional maturation that allowed force analysis studies upon electrical stimulation. We proved that DMD-derived MYOrganoids recapitulate salient features of DMD pathogenesis, such as membrane fragility and force drop upon eccentric contraction. We then employed our system to evaluate the therapeutic efficiency of micro-dystrophin, a gold standard in the AAV-gene therapy, as a proof of concept. Interestingly, we showed that DMD MYOrganoids, subjected to eccentric contraction, better retain muscle force after micro-dystrophin treatment. This indicates that our system has the potential to be a valuable human *in vitro* counterpart to animal *in vivo* studies, to test the real efficacy of gene therapy and alternative treatments.

197 CRISPR-Mediated Insertion of a Transferrin Receptor-Targeted *GAA* Transgene into Hepatocytes Provides Effective, Long-Lasting Treatment of Muscles and the Central Nervous System in Neonate and Adult Pompe Disease Mice

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Pompe disease is a lysosomal disease caused by mutations in the acid alpha-glucosidase (*GAA*) gene. Loss of GAA results in toxic accumulation of lysosomal glycogen in tissues. Pompe disease manifests as muscle weakness, respiratory failure, increased risk of aneurysm and stroke, and in severe cases intellectual disability. The prevalence of central nervous system (CNS) manifestations of Pompe disease is much higher in severe, late-onset Pompe disease and infantile-onset Pompe disease. The current standard-of-care enzyme replacement therapy treats the peripheral pathologies in Pompe patients, but this therapeutic requires lengthy biweekly infusions, high doses of drug as GAA has weak tropism to muscle tissues and does not treat the central nervous system. Liver-directed AAV gene therapy may improve muscle phenotypes due to high continuous expression of GAA but is also unlikely to robustly treat the CNS as GAA poorly crosses the blood brain barrier (BBB). Additionally, conventional AAV gene therapy utilizes AAV episomes to express GAA, which will be lost as hepatocytes divide due to liver growth or damage. As such, episomal gene therapy has limitations for treating the significant pediatric population of Pompe patients. Here, we present: **(1)** A rapid *in vivo* screen of 31 anti-human TfR1 antibodies in *TfRC* humanized mice for their ability to deliver GAA across the BBB. Based on hydrodynamic delivery, this screening strategy effectively identified top antibodies which delivered GAA

to the CNS without needing to generate AAV or purified protein. In contrast to some previous literature on anti-TFR antibodies, we found that high-affinity antibodies rather than low-affinity antibodies imparted the best delivery to both the CNS and muscles. **(2)** A CRISPR-mediated insertion approach for both infantile and late-onset Pompe disease, where a transgene is inserted into the first intron of the albumin locus to express anti-hTfR1:GAA under control of the endogenous albumin promoter. The insertion template DNA encoding anti-hTfR1:GAA is delivered by an AAV, and an mRNA encoding Cas9 and a gRNA specific for a site in the 1st intron of albumin is delivered by a lipid nanoparticle. Insertion of anti-hTfR1:GAA in this manner provided robust expression from a single dose, and **complete or near-complete clearance of pathogenic glycogen from the heart, skeletal muscles, the dorsal root ganglion, spinal cord, and the brain** in Pompe disease model mice within 3 weeks after delivery. We have previously presented that CRISPR-mediated insertion of muscle targeted antibody-GAA fusion (anti-CD63:GAA) in **neonatal mice** resulted in stable expression throughout a 15 month study, whereas expression from episomal AAV decreased over time. Here, we further show that this approach is a platform which can be translated to across transgenes, as well as to non-human primates. By administering a BBB-crossing GAA fusion via CRISPR-mediated insertion, this approach has the potential to be a true single-dose therapy with life-long efficacy for both late-onset and infantile-onset Pompe disease.

198 Improved AAV-Mediated Systemic Gene Therapy to Striated Muscles through Increased Tissue Perfusion

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One of the most common and efficient gene delivery vectors is adeno-associated virus (AAV). For many disorders, systemic delivery of gene therapy is necessary to gain the desired distribution of vector and achieve a therapeutic effect in target tissues. As this typically requires high doses of AAV which can have safety concerns, methods to improve efficacy and reduce gene therapy doses are highly desirable. A potential solution for certain diseases could be to induce a rebalancing of tissue perfusion in the body to favor specific target organs via low-intensity acute aerobic exercise prior to systemic intravenous (IV) AAV administration. To test this, we assessed systemic IV delivery of the marker gene vector AAV9-CBA-Luciferase (AAV9-Luc) at a dose of 5 ×1012 vg/kg via the jugular vein in adult wild-type C57/BL6 mice following 1) no intervention, 2) a single bout of acute, low-intensity, aerobic treadmill exercise, 3) a single bout of acute, high-intensity, aerobic treadmill exercise, and 4) administration of Dobutamine (a beta-1 agonist drug) to provide increased ventricular contractility and, decreased afterload as a potential exercise surrogate. Vital measurements including heart rate, perfusion index, temperature, and oxygen saturation (SpO2) were acquired immediately following the intervention and for up to 10 minutes following AAV administration. Non-invasive, in vivo bioluminescence imaging of the luciferase reporter was performed every other week until the completion of the study at 6 weeks post-administration. Full necropsies were performed and tissues were being assessed for vector genome (vg) uptake, transgene transcription levels, luciferase activities, and immunofluorescence imaging to determine the location and quantification of expression within tissues. Our results show that the low and high intensity exercise and dobutamine groups all display a significant increase in tissue perfusion and heart rate at the time of AAV IV administration (10 minutes post-intervention). Based on our tissue assessments thus far, in vivo bioluminescence imaging data revealed an increase in luciferase expression based on detected relative luciferase units in the low intensity exercise and dobutamine groups. Luciferase RNA transcript levels were found to be significantly increased in the heart, liver, brain cortex, gastrocnemius, and extensor digitorum longus (EDL) in the low intensity group, the quadriceps in the high intensity exercise group, and the heart, liver, and tibialis anterior in the dobutamine group each as directly compared to no-intervention, AAV treated control mice. Ongoing studies include completion of vector genome and RNA transcript assessments, evaluations of luciferase expression within tissue sections, and luciferase protein expression levels. Additional mice are being added to each cohort to evaluate the effects of each intervention on AAV receptor expression. Our results suggest that an increase in heart rate and tissue perfusion caused by either one bout of exercise or dobutamine administration can significantly increase the effectiveness of systemic AAV gene therapies targeting striated muscles.

199 PGN-EDODM1 Nonclinical Data Demonstrate Potential for Meaningful Impact in Myotonic Dystrophy Type 1 (DM1): Support for Phase 1 Clinical Trial Design

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Background: PepGen's Enhanced Delivery Oligonucleotide (EDO) cell-penetrating peptide technology is engineered to optimize tissue delivery and cellular uptake of therapeutic oligonucleotides. Delivery of oligonucleotides to affected tissues is a major challenge that limits their efficacy. PGN-EDODM1 is being evaluated for the treatment of DM1. PGN-EDODM1 binds to the pathogenic trinucleotide repeat expansion in *dystrophia myotonica protein kinase* (*DMPK*) mRNA and reduces sequestration of muscleblind (MBNL) proteins to the myonuclear RNA foci through a steric blocking mechanism. Release of MBNL1 protein is hypothesized to correct DM1 spliceopathy; a central cause of DM1. **Objectives:** To develop a nonclinical dataset, including in vitro and in vivo pharmacology studies, to support initiation of clinical studies of PGN-EDODM1. **Methods:** In vitro studies: DM1 patient derived myoblasts with 2,600 CTG repeats and control myoblasts were treated with PGNEDODM1, and nuclear RNA foci profiles, splicing events and levels of *DMPK* transcript were evaluated after 24 hours. In vivo studies: PGN-EDODM1 or vehicle control were administered

intravenously to the DM1 mouse model HSA^{LR} (containing ~220-250 CTG repeats in the *HSA* gene). Splicing events and myotonia were measured, as well as effects on select off-target transcripts containing CUG repeats. Long-term effects of PGN-EDODM1 were investigated by splicing changes over 24 weeks after a single dose. Studies in non-human primates (NHPs): Three doses of 10, 30 or 60 mg/kg of PGN-EDODM1 were administered every two weeks or every four weeks (60 mg/kg only) to NHPs and one week following the final dose, *DMPK* transcript levels were evaluated by RT-PCR and normalized to *RPLP0*. **Results:** PGN-EDODM1 administration resulted in a dose-dependent reduction in toxic RNA foci and correction of mis-splicing in DM1 differentiated myotubes. *DMPK* levels remained unchanged in differentiated myotubes after administration of PGN-EDODM1. In the HSA^{LR} model, a single dose resulted in high muscle concentrations of PGN-EDODM1, resolution of myotonia at 2-weeks postdose and dose-dependent correction of mis-splicing. Correction of splicing has a long duration of action persisting up to 24 weeks. Off-target effects were not observed in other transcripts containing more than 10 CUG repeats in mice. In NHPs, PGN-EDODM1 resulted in no decreases in *DMPK* expression. **Conclusions:** There are no approved therapies for DM1. PGN-EDODM1 shows considerable therapeutic potential both in vitro and in vivo, including correction of myotonia in an established mouse model of DM1 and correction of key mis-spliced transcripts without the degradation of *DMPK* (or *HSA*) both in vitro and in vivo. Nonclinical data of PGN-EDODM1 support a Phase 1 single-ascending dose study in adults with DM1 that we plan to initiate in 2023. Details of the clinical trial design will be presented.

Breaking Gene Editing and Other Barriers for Cardiovascular and Pulmonary Diseases

200 Gene Transfer Efficiency in Models of Increased Secreted Airway Mucins

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Airway mucus is essential for lung health by trapping inhaled small particles and transporting them out of the lung via mucociliary transport. Viral-based vectors, such as adenovirus, adeno-associated virus, and lentivirus, have all shown promise at delivering CFTR cDNA (gene addition) or gene editing machinery, such as CRISPR-Cas9, to airway epithelial cells. Given the role of airway mucus in host defense, one can easily envision mucus as a possible barrier to efficient viral vector mediated gene transfer, particularly in CF airways. Many studies use CF sputum as a model for the mucus barrier; however, this model may not accurately represent the dynamic *in vivo* environment. The goals of this study are to: 1) determine the degree to which mucus prevents viral vector mediated transduction; 2) advance physiologically relevant *in vitro* and *in vivo* models of mucus barriers to gene therapy reagents; and 3) determine if mucolytic interventions can improve vector transduction of airway epithelial cells. We first tested an ex vivo model using tracheal explants from CF and non-CF pigs. Explants maintained on surgifoam retain their tissue and cellular morphology for at least four weeks; additionally, they remain capable of mucus secretion from both goblet cells and submucosal glands. Explants were treated with methacholine, a cholinergic agonist that stimulates glandular mucus secretions. We observed that Ad5 vector expressing mCherry readily transduced methacholine treated tracheal explants. Using microscopy and ImageJ, we quantified Ad transduction following treatments with the reducing agent dithiothreitol (DTT) or the airway surfactant lysophosphatidylcholine (LPC). The addition of LPC had the greatest impact on gene transfer efficacy. We next investigated Ad5 transduction in a mouse model of goblet cell metaplasia. IL-13 treatment induces goblet cell metaplasia and increases mucus secretion. IL-13 vs saline pre-treated mice were instilled intratracheally with Ad5 expressing firefly luciferase ± LPC. Similar to the results in methacholine treated explants, the IL-13 treatment group did not result in a decrease of transgene expression; however, the addition of LPC improved transduction. Although these models do not fully recapitulate the complex inflammatory environment of the CF lung these results do suggest that mucus hypersecretion is not a significant impairment to Ad5 transduction, especially in conjunction with an LPC vehicle. Ongoing studies are investigating the impact of mucus hypersecretion on lentiviral and AAV-based vectors.

201 *SCN10A-short* Gene Therapy for the Treatment of Cardiac Conduction Disorders

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The cardiac voltage-gated sodium channel $Na_V1.5$ is an important therapeutic target to treat cardiac conduction disorders, such as Brugada syndrome and isolated cardiac conduction defect. However, the large size of *SCN5A* encoding $Na_V1.5$ imposes a substantial challenge to gene therapeutic interventions. Alternatively, small proteins that modulating sodium current can be good gene therapeutic targets. We previously reported that a naturally occurring short transcript encoding a C-terminal portion of neuronal *SCN10A (SCN10A-short)* increases sodium current in an *SCN5A*-dependent manner. In the present study we developed an AAV-based *SCN10A-short* gene therapy for the treatment of cardiac conduction disorders. First, we studied the effect of *SCN10A-short* overexpression in wild-type mouse cardiomyocytes using single cell patch-clamp. *SCN10A-short* overexpression resulted in a significantly larger sodium current and faster action potential upstroke. We next studied whether *SCN10A-short* gene therapy could improve cardiac conduction in $Na_V1.5$ haploinsufficient mice. Heterozygous *Scn5a* mutant mice were treated with either moderate dose or high dose *SCN10A-short* gene therapy and mice that received treatment with moderate dose GFP gene therapy were used as control. Two weeks post injection, hearts were isolated and perfused on a Langendorff setup, where optical mapping was performed to measure epicardial conduction. Compared with controls, mice receiving moderate dose *SCN10A-short* gene therapy showed significantly increased conduction velocity in the left ventricle. Strikingly, the high dose *SCN10A-short* gene therapy led to faster

conduction velocities in both ventricles, which was comparable to wild-type hearts. Electrocardiogram (ECG) analyses revealed significantly shorter QRS duration which is similar to wild-type, in high dose *SCN10A-short* treated animals as compared with controls, reflecting the improved cardiac conduction. To validate our gene therapy in human cells, we also tested the effect of *SCN10A-short* overexpression in cardiomyocytes obtained from human induced pluripotent stem cells. Single cell patch-clamp experiments showed a significantly higher action potential upstroke velocity compared with GFP-treated control cells, demonstrating a substantial increase in *SCN10A-short* induced sodium current in a human background. Finally, we tested the *in silico* effects of *SCN10A-short* on human ventricular conduction velocity and excitability in case these were affected by a severe loss-of-function mutation in *SCN5A*. In a linear strand of left ventricular cells, the conduction slowing and excitability loss caused by the mutation were largely restored with *SCN10A-short.* In a linear strand model with multiple branches, the mutation-induced partial conduction block in the branched part of the strand was fully restored by the application of *SCN10A-short.* In summary, our results highlight the potential of *SCN10A-short* gene therapy as an effective therapeutic intervention to normalize cardiac conduction in inherited and acquired conduction disorders.

202 EV-AAVs as a Novel Gene Delivery Vector to the Heart: New -Evaluations for EV-AAVs and Free AAV Separation

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Introduction: Adeno-associated viruses (AAVs) are one of the best vectors to deliver genes to the heart due to high transgene expression and safety in clinics. However, pre-existing AAV neutralizing antibodies (NAbs) in patients binds to AAVs, prevent efficient gene transduction and limits the number of patients eligible for gene therapy. Here, we have shown that extracellular vesicle-encapsulated AAVs (EV-AAVs) are a superior cardiac gene delivery vector that delivers more genes and offers higher NAb resistance comparted to free-AAVs. Presence of free AAV contaminants in EV-AAV preparations may reduce effective EV-AAV dosing, lower their NAb resistance, and cause unwanted side effects. Therefore, novel methods are required to efficiently remove free AAVs from EV-AAV preparations to improve their efficacy. **Methods and Results:** We use a two-step iodixanol density-gradient ultracentrifugation method (UC+DG) to purify EV-AAVs from free of AAV contamination. We comprehensively characterized the isolated EV-AAVs for their morphology, presence of EV specific markers, size, concentration and AAV genome titer using WB, nano (EV)-flow cytometry, qPCR, TEM, DLS. To develop a scalable EV-AAV production for clinical applications, we compared the UC+DG method with two new, fundamentally distinct technological solutions — a nanofluidic deterministic lateral displacement (nanoDLD) device and size-exclusion chromatography (SEC). We ranked the techniques by their output for EV-AAV enrichment, runtime, reproducibility, processing volume and ease of use. We comprehensively compared all 3 technologies for EV-AAV isolation using a combination of experimental and computational modeling and report that ~25-50% of AAV vector genome is secreted via tetraspanin positive, NAb-resistant, ~80nm EV-AAVs. In addition, our results demonstrated NAb evasion and therapeutic efficacy of EV-AAVs. Intramyocardially injected EV-AAV9-SERCA2a, a known therapeutic cardiac gene, to post- infarcted (LAD ligated), pre-immunized mouse hearts significantly improved ejection fraction and fractional shortening compared to free AAV9-SERCA2a delivery. Using PKH67-fluorescent dye or pH sensitive (CypHer) dye, we show that EV-AAVs were internalized into acidic endosomal compartments before reaching the nucleus in human cardiomyocytes.**Conclusion:** We confirmed efficient separation of EV-AAVs from free AAVs using UC+DG as EV gold standard method, and we provided valid and alternative EV-AAV isolation technologies highly adoptable for clinical use. We also show significantly higher potency and therapeutic efficacy of EV-AAVs compared to free AAVs. Our results establish the potential of EV-AAVs as a state-of-the art gene delivery vector to treat heart failure and our study demonstrates a successful approach to technology development for isolating EV-AAVs providing innovative solutions to a critical barrier in gene therapy.

203 Proliferation and Differentiation Potential of CFTR-Expressing Epithelial Cells in Mouse Airways

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Cystic fibrosis (CF) is an inherited disease caused by mutations of *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)* gene. Lung diseases is the main cause of death in CF patients. Genetic therapy targeting airway epithelial stem cells thus holds considerable promise for permanent cure of CF disease. CFTR is heterogeneously expressed in distinct airway epithelial cell types including basal cells, secretory cells, and pulmonary ionocytes. CFTR protein was largely undetectable in airway basal cell membranes, however, a subset of airway basal cells destined to be secretory cells expresses CFTR mRNA. The role of these CFTR-expressing basal cells in airway epithelial regeneration and maintaining physiologic homeostasis of airway epithelium remain unclear. To better define the role CFTR-expressing basal cells in the airway epithelial regeneration, a mouse model capable of fate mapping CFTR-expressing cell types was created by inserting an ires-CreERT2 cassette into the 3'UTR of the *Cftr* gene. Rosa26nTnG::*Cftr*-ires-CreERT2 (Rosa::*Cftr*Cre) reporter transgenic mouse were created fate map CFTR expressing cells. CFTR-expressing cells in Rosa::*Cftr*Cre mice when genetically labeled with tamoxifen converts nuclear tdTomato (nT) to nuclear GFP (nG) expression. The CFTR-expressing cell lineages could be identified by immunolocalization of nG and airway epithelial cell markers in native mouse airway and using an air-liquid interface (ALI) cultures generated from primary surface tracheal basal cells and submucosal gland (SMG) cells of Rosa::*Cftr*Cre mouse airways. Results showed that rare GFP-labeled cells were mainly confined to the proximal tracheal epithelia and SMGs in the trachea of transgenic mice. Labeled secretory and intermediate basal cells were absent in the trachea and only rare club labeled cells were present in the intralobar

airways. Notably, GFP-labeled cell colonies were observed along the distal tracheal epithelia in mice following naphthalene-induced airway epithelial injury. In addition, a high frequency of GFP+ Type II alveolar cells were observed lung paryncyhma. Immunolocalization analysis demonstrated that majority of GFP-labeled cells also express ionocyte marker Bsnd and FoxI1 in the proximal trachea, and surfactant protein-C in alveoli. The observation in native transgenic mouse trachea was further corroborated in ALI cultures generated from cells of surface airway epithelium (SAE) and SMGs. Fate mapping of CFTR expressing during *in vitro* airway epithelial cell differentiation in the presence of hydroxytamoxifen demonstrated that >80% of GFP-labeled lineages expressed ionocyte markers Bsnd and FoxI1, but not major epithelial cell types such as ciliated, secretory and basal cells. Notably, SMG-derived progenitors generated significantly more lineage-labeled cells than SAE progenitors, the majority of which also expressed Bsnd and FoxI1, suggesting that SMGs may be enriched in progenitors of pulmonary ionocytes. Interestingly, FACS sorted GFP-labeled epithelial cells from ALI cultures of primary airway epithelial cells Rosa::*Cftr*Cre mice demonstrated the ability to differentiate into airway ciliated, secretory, and ionocytes, when they were reseeded on ALI membrane for epithelial cell differentiation. These data suggest that a subset of CFTR-expressing basal cells and/or club secretory cells are multipotent progenitors. Further studies of the GFP-labeled cells at single cell resolution may help to better define pulmonary CFTR-expressing progenitors in airways.

204 AAV Gene Editing Extends Survival in a Mouse Model of Surfactant Protein B Deficiency

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Surfactant protein B (SP-B) deficiency (OMIM#265120) is a monogenic disorder that demonstrates lethality in the neonatal stage. Infants diagnosed with this disorder display respiratory distress and survival is limited to the 1-year post birth. The disorder is caused by a mutation in the *SFTPB* gene and leads to lung atelectasis due to improper surfactant production. Current treatment options include exogenous drug delivery, a transient manner to treat the disease, but successful long-term treatment is dependent on a lung transplant. Due to the risks involved with transplantation, alternative treatments are needed for this afflicted pediatric population. One proposed concept is treating patients with gene therapy. Viral based gene therapy vectors, such as adeno-associated viral vectors (AAV), have gained popularity in the past decade, due to their strong safety and efficacy profile. We previously demonstrated that survival can be significantly extended in surfactant protein B knockout mice when an engineered AAV6.2FF vector expressing the SP-B gene is administered into the lungs. With this gene addition platform survival can be extended to 294 days, however further advancements to this vector-based gene therapy are needed to address the waning expression observed. Due to the immunity developed upon AAV exposure, repeat administration with the same capsid has been shown to be ineffective. Therefore, a more permanent form of gene therapy is required to obtain sustained expression in this pediatric population, ensuring long term expression in the developing organ targeted without expression dilution overtime from cell turnover. Gene editing tools allow for permanent gene correction and here we investigated whether our AAV-CRISPR platform could extend survival in SP-B -/- mice. Using our AAV-CRISPR platform we achieved gene insertion efficiencies from 6% up to 25% in lung epithelial cells that increased in a dose dependent manner. Here we applied both the donor and nuclease templates in separate AAV capsids with the aim of permanently modifying the genome to insert a functional SP-B gene. Treatment groups were as follows: control groups of untreated mice either on or off dox (n=6 for each) and a group of mice delivered the gene addition form of the SP-B platform (n=4), in addition to, gene editing groups that consisted of mice delivered either 1e12 vg of the donor template (n=3) or 1e12 vg of donor template and 5e11 vg of the nuclease (n=6). In this proof-of-concept trial, we were able to see significant extension in median survival (P=<0.0001) in the gene corrected mice administered both donor and nuclease template. Median survival for this groups was approximately 16 days in comparison to the 6-day survival seen in the donor template only group. As of February 1st, 2023, there is still one mouse remaining in the donor and nuclease gene edited group, still surviving at 231 days. This data demonstrate the safety and efficacy of this AAV-CRISPR platform for lung gene editing and establishes a baseline for further improvements of this promising gene editing platform.

205 LX2020-An AAV Based Gene Therapy Improves the Arrhythmogenic Right Ventricular Cardiomyopathy Phenotype in a Severe Mouse Model Harboring Human PKP2 Mutation

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a fatal genetic-based cardiac disease that increases risk of sudden cardiac death and can progress to heart failure. Clinical studies have established that mutations in the desmosomal gene, plakophilin-2 (PKP2), represent the most frequently mutated gene (>60%) in ARVC. To date, no effective treatment or cure is known to exist for ARVC. Through CRISPR-Cas9 methods, a novel knock-in mouse model was generated harboring a human equivalent PKP2 splice site mutation (IVS10-1 G>C) that impacted PKP2 RNA splicing, with consequences on cardiac PKP2 protein quality (presence of mutant protein) and levels (decreased), resulting in all classic postnatal ARVC disease features in PKP2 homozygous mutant (Hom) mice. LX2020 is a novel AAVrh.10 based gene therapy designed to overcome cardiac deficits in PKP2 levels via the cardiac expression of human PKP2 protein and is being developed for patients with ARVC due to a mutation in

the PKP2 gene. Research grade LX2020 was leveraged to establish proof-of-principle based expression of hPKP2 in the heart that when administered at early postnatal stages in PKP2 Hom mice translated to improvements in cardiac dimensions and function in adult PKP2 Hom mice. To test the efficacy of clinical grade LX2020 in mild and severe ARVC disease settings, LX2020 was produced using the LEXEO manufacturing process and administered intravenously to young postnatal Day 5 mice as well as 3-4 week-old diseased adult PKP2 Hom mice. A dose-dependent increase in hPKP2 expression was seen in young PKP2 Hom mouse hearts that superseded endogenous wild-type (WT) mouse levels. LX2020 administration in young postnatal PKP2 Hom mice translated into restoration of cardiac PKP2 levels and prevention of cardiac cell-cell desmosome and junctional protein deficits in adult PKP2 Hom mice. Histological analysis of adult PKP2 Hom heart tissue treated with LX2020 revealed substantial prevention of left and right ventricle chamber dilatation and remodeling (fibrosis), when compared to controls, with PKP2 Hom hearts treated with the highest dose of LX2020 being indistinguishable from WT controls. Most importantly, LX2020 administration in severely diseased adult PKP2 Hom mice also translated to a dose-dependent increase in hPKP2 expression in PKP2 Hom hearts that resulted in reduction in cardiac cell-cell junction deficits as well as improvements in left and right chamber dimensions and function as well as survival. Collectively, these data indicate that LX2020 overcomes cardiac PKP2 deficits in ARVC settings by driving human PKP2 expression and that this is sufficient to prevent and stabilize the progression of a rapid ARVC disease in young and adult PKP2 Hom mice, respectively. LX2020 thus represents a potentially effective clinically relevant gene therapy approach to treat patients with ARVC disease.

206 Efficient *In Vivo* Base Editing Prevents Hypertrophic Cardiomyopathy in Mice

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Dominant missense pathogenic variants in cardiac myosin heavy chain cause hypertrophic cardiomyopathy (HCM), a currently incurable disorder that increases risk for stroke, heart failure, and sudden cardiac death. We assessed two different genetic therapies, an adenine base editor (ABE8e) and a potent Cas9 nuclease delivered by AAV9, to prevent disease in mice carrying the heterozygous HCM pathogenic variant myosin R403Q. One dose of dual-AAV9 vectors, each carrying one half of RNA-guided ABE8e, corrected the pathogenic variant in ≥70% of ventricular cardiomyocytes and prevented fibrosis while maintaining durable, normal cardiac structure and function. An additional dose provided more editing in the atria but also increased bystander editing. Single-cell RNA-seq confirmed that cardiomyocytes in animals receiving base editor treatments had a significantly reduced signature of hypertrophy-associated expression profiles. Due to the use of a cardiomyocyte-specific promoter in the AAV9 vector, genome editing was restricted to the heart. CIRCLE-seq identified sites of off-target base editing using this genome editing strategy. Off-target editing following AAV9 delivery at five sites was observed to increase during the seven-month follow up, reaching over 8% at one site. However, off-target base editing did not appear to reduce the fitness of the heart or mouse. AAV9 delivery of RNA-guided S. aureus Cas9 nuclease effectively inactivated the pathogenic allele, albeit with dose-dependent toxicities, necessitating a narrow therapeutic window to maintain health. High doses of Cas9 nuclease led to the disruption of the non-targeted wild type allele over the course of the seven-month follow up, and these animals displayed reduced heart function. These preclinical studies identify limitations and demonstrate considerable potential for single-dose genetic therapies to correct or silence pathogenic variants and prevent the development of HCM.

Modulating and Engineering Immune Responses to Gene Therapy and Vaccines

207 Combination Immunosuppression Prevents Toxicity and Increases Liver Transduction in Cynomolgus Macaques Administered with High Dose AAV Vector

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Adeno-associated virus (AAV) based genetic medicines have been transformational in treating several genetic disorders. The widespread adoption of AAV vectors for in vivo gene delivery was based on the safety and efficacy of vector in targeting multiple tissues. However, clinical trials using AAV gene therapies have observed severe adverse events (SAE's) owing to the innate and adaptive immune responses against the AAV capsid antigens and/or the transgene products. Furthermore, recent clinical trials have observed complement activation, thrombotic microangiopathy/Hemolytic Uremic Syndrome (aHUS), and hepatotoxicity in subjects intravenously administered high AAV vector doses. The use of prophylactic or reactive glucocorticoids has been ineffective in completely ablating or reducing high-dose vector induced toxicity. Instead, intervention with additional

immunosuppressants (IS) have provided clinical benefit in reducing toxicity associated with complement activation. Here we studied the effect of different IS drug combinations to reduce toxicity following intravenous administration of high dose (5E13 vg/kg) vector in Cynomologus Macaques. Animals (N=3/group) received either an AAV9 vector expressing GFP from a ubiquitous promoter or vector along with one of the following IS regimens: 1) methylprednisolone (MP), 2) MP and rapamycin, 3) MP, rapamycin and rituximab (triple IS). The study was terminated 56 days post vector administration and tissues were collected for further evaluation. During the in-life phase of the study, animals that received vector without any IS were lethargic and demonstrated signs of hindlimb dysfunction 30 days post vector infusion. In contrast, animals that received triple IS showed normal behavior with no functional deficits. The triple IS regimen prevented hepatotoxicity with no significant transaminitis. The triple IS treated animals had reduced DRG toxicity and cardiac troponin I levels were normal. Immunotoxicity assessment demonstrated reduced innate and adaptive immune activation in triple IS regimen treated animals. First, there was a 100x reduction in anti-AAV9 neutralizing antibodies and a significant reduction in anti-AAV9 IgG and IgM binding antibodies. Serum levels of C5b9 and Bb were lower suggesting reduced complement activation in these animals. Next, immunophenotyping revealed significant decreases in B cells, T cells, and NK cells in triple IS treated animals when compared to non-IS controls. IFN-g ELISPOT assays revealed reduced transgene-specific cytotoxic T cells in the triple IS treated group. Finally, immunosuppressed animals had delayed blood vector clearance and higher liver transduction with increased transgene expression. Overall, the findings here support the use of these or similar regimens to reduce toxicity and increase safety of AAV gene therapy in the clinic.

208 Inhibition of AAV-Specific Adaptive Immune Response by Co-Stimulation Blockade with CTLA4-Ig Fusion Protein

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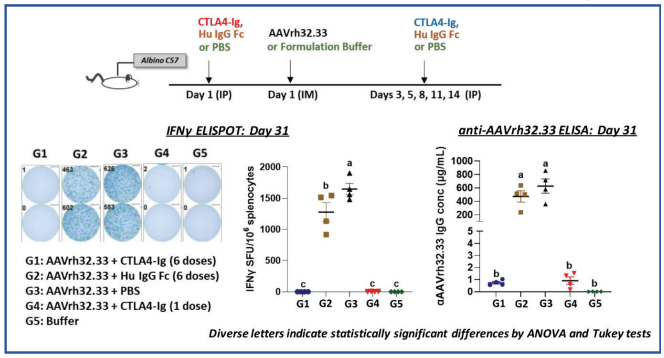
One of the major limitations to the use of Adeno-Associated Virus (AAV) gene therapy is the adaptive immune response that contributes to immune related adverse events and prevents AAV redosing. Thus, there is a need for efficient strategies to reduce or prevent the generation of neutralizing antibodies and to inhibit T cell responses. CTLA4 is an inhibitory immune checkpoint receptor that competes with CD28 for binding to CD80/CD86 on antigen presenting cells and consequently limits T cell co-stimulation. A recombinant protein that consists of the CTLA4 extracellular domain linked with the Fc domain of a human immunoglobulin (CTLA4-Ig) has shown to limit adaptive immune responses in preclinical models. CTLA4-Ig variants like Abatacept and Belatacept have been approved for clinical use in patients with autoimmune conditions or in organ transplant settings. Previous evidence has shown that CTLA4-Ig can modulate the immune response in the context of AAV8 or rAAV2/1 vectors, when delivered as a transgene or in combination with other immune modulators (Adriouch et al. 2011; Zhong et al. 2022). In this study we evaluated the effect of CTLA4-Ig on the adaptive immune response induced by AAVrh32.33, a highly immunogenic AAV variant that generates strong B and T cell responses when administered intramuscularly in C57BL/6 mice (Mays et al. 2009). Single administration of CTLA4-Ig on the same day of AAVrh32.33 injection (day 1) was compared with a multiple dose regimen where another five CTLA4-Ig doses were inoculated separated 2-3 days until day 14. A first group of control mice received AAVrh32.33 and six doses of a human Ig Fc domain while other control animals were given PBS and formulation buffer. A single dose of CTLA4-Ig was sufficient to abrogate the generation of AAVrh32.33-specific CD8$^+$ T cells 30 days after vector dosing in 100% of the treated mice, as demonstrated by both IFNγ ELISPOT and flow cytometry staining of antigen experienced AAV dextramer positive T cells. AAVrh32.33-specific serum IgG antibodies were significantly inhibited at this time point by both CTLA4-Ig dose schedules. Interestingly, serum IgM antibodies specific for AAVrh32.33 measured at an earlier time point (day 9) were also significantly decreased in the animals treated with either one or six doses of CTLA4-Ig. Multiple CTLA4-Ig doses were required to maintain significantly reduced AAV-specific CD8$^+$ T cell frequencies and IgG antibody levels in 75% of the treated mice 90 days after vector inoculation. No difference in luciferase transgene expression was detected in the vector injection site (right tibialis anterior muscle) between any of the treatment groups throughout the study. In conclusion, co-stimulation blockade with CTLA4-Ig represents a promising immune modulation strategy for inhibiting AAV induced B and T cell responses with the potential to reduce immune related adverse events and facilitate re-dosing.



209 Engineered Human B Cells Respond to Antigen-Specific Immunization in a Tonsil Organoid Model

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B cells are emerging as an exciting immune therapy, with the potential to combine long-term expression of custom therapeutic antibodies with other functions of the humoral immune system. For example, memory B cells undergo clonal expansion in response to antigen, creating a system that is intrinsically self-tuning, while somatic hypermutation facilitates the ongoing evolution of antibodies. Most of these features depend on expression of the antibody from the natural immunoglobulin locus. We have previously described a genome editing strategy to reprogram the heavy chain locus of human B cells

to express custom heavy-chain only antibodies (HCAbs) based on IgG1. These antibodies mimic a natural antibody type expressed by camelids and can be engineered with a variety of antigen-recognizing domains, including VHHs, scFvs, and receptor domains. Engineered HCAbs maintain the antigen specificity of their inserted cassette and the edited cells support a range of normal B cell functions during *ex vivo* differentiation. In addition, sequence changes characteristic of somatic hypermutation develop in HCAb-edited B cell lines over time. However, a lack of robust *in vivo* models makes it challenging to confirm that engineered human B cells will respond appropriately to antigens in an immunologic context. Indeed, such studies typically rely instead on editing mouse B cells. Here, we report the use of a tonsil organoid model that represents a 'human immune system in a dish' to evaluate HCAb-engineered human B cells. Exposure of such organoids to antigen induces clonal expansion of antigen-specific B cells and drives both class-switch recombination and somatic hypermutation (Wagar *et al.* Nat Med 2021). To assess the functionality of our edited cells, we reprogrammed tonsillar B cells to express an anti-HIV HCAb, based on the broadly neutralizing llama VHH, J3. Tonsil organoids were supplemented with J3-edited B cells and immunized with recombinant HIV gp120 protein plus adjuvant. The frequency of edited B cells increased after gp120 exposure, but not following a control immunization with PE, indicating an antigen-specific response. Further, gp120 immunization increased the frequency of mutations in the J3 sequence at AID hotspot motifs, suggesting somatic hypermutation. Several of these mutated clones were expanded up to 600-fold in immunized vs. non-immunized organoids and expansion of unique clones was observed across multiple donors, suggesting that these were *de novo* mutations that expanded after immunization. In sum, these data provide evidence that HCAb-engineered human B cells can functionally respond to their specific antigen, undergoing clonal expansion and somatic hypermutation. To our knowledge, this is the first report of an antigen-specific immune response by engineered human B cells, suggesting that the tonsil organoid model will be a valuable tool to assess engineered B cell functionality in the absence of a suitable animal model.

210 Newly Engineered IgM and IgG Cleaving Enzymes for AAV Gene Therapy

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Clinical translation of systemic AAV-based gene therapies is restricted in scope due to preexisting immunity to natural AAV serotypes in prospective patients. In addition, humoral immunity to AAV vectors has been implicated in triggering complement activation often resulting in adverse clinical events. Strategies to mitigate undesirable side effects and antibody-mediated AAV neutralization have included plasmapheresis, B cell depletion (e.g., Rituximab), complement inhibition (e.g., Eculizumab), and most recently, IgG-degrading enzymes such as IdeS and IdeZ. Despite these efforts, the negative impact of IgM, a critical component of humoral immunity to AAV vectors remains unaddressed to date. Here, we describe newly engineered enzymes that can selectively cleave IgM and/or IgM + IgG that could potentially impact clinical AAV gene therapy. The novel IgM-cleaving enzyme (IceM) efficiently cleaves IgM with a high degree of specificity. IceM cleaves human and NHP IgM, but not IgM from other species such as dog, pig, or mouse. Further, IceM cleaves IgM from the surface of B lymphocytes *in vitro*. To expand on the clinical applicability of IceM, we further engineered a novel IgM + IgG cleaving enzyme (IceMG) that effectively cleaves antibodies in human and NHP sera. Notably, both IceM and IceMG block complement activation elicited by either AAV9 capsids or Platelet factor 4(PF4)/heparin complexes in human sera. In mice passively immunized with pooled human sera, we demonstrate efficient and rapid (within 24h) clearance of circulating human IgM with a single intravenous dose of recombinant IceM. Results demonstrating clearance of neutralizing human IgM + IgG upon dosing recombinant IceM and IceMG accompanied by rescue of AAV transduction in passively immunized mice will be presented. Taken together, our newly engineered enzyme platform has implications for expanding the patient cohort and vector redosing in AAV gene therapies.

211 A Biosynthetic Approach for CpG Methylation of AAV Expression Cassettes

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Adeno-associated virus (AAV) based therapies have the potential to provide transformative treatments for genetic diseases. However, immune responses induced by AAV remain a significant barrier. Immune responses potentiated by binding of unmethylated CpG dinucleotides in AAV vector genomes to the microbial DNA pattern recognition receptor TLR9 are thought to play a role in the loss of therapeutic transgene expression due to the destruction of the transduced cells by cytotoxic T cells (CTLs). Current strategies to reduce TLR9 activation include codon modifications to remove CpGs in the transgene sequence. However, one risk of modifying codon usage is the potential for trans protein misfolding when wild-type translational kinetic are changed. We are investigating strategies that increase vector genome CpG methylation to a level comparable to methylation patterns in humans to remove the main stimuli for TLR9 activation. Our strategy to increase AAV genomic CpG methylation employs an efficient methyl transferase (MT), MSssI from *Spiroplasma*, at two steps in the vector production process. First, because vector plasmid encoded expression cassette DNA can be directly packaged into capsids, vector plasmids were methylated prior to transfection into production cells. Second, because expression cassettes are also generated through replication, MT was integrated into production cells and expressed after triple transfection. We previously described an acellular method using MT, which efficiently methylated the CpGs in AAV vector plasmids and significantly decreased TLR9 activation in HEK reporter cells. Plasmid methylation also decreased GFP transgene expression when transfected into HeLa cells, compared to non-methylated controls. Furthermore, acellular plasmid methylation is not scalable for vector manufacturing. To address these two challenges, we hypothesized that i) promoter methylation silenced transgene expression, and ii) that genomic MT integration in *E.Coli* would better facilitate scalable production of methylated AAV vector plasmids. Here, we compared acellular methylation of CpG-containing promoter driven GFP vector plasmid (pAAV2-CAG>GFP) and CpG-free promoter driven GFP vector plasmid (pAAV2-CpGfreeEF1a>GFP) and found that MT

efficiently methylated both vector plasmids. We then transfected these plasmids, and non-methylated counterparts, into HEK293. After staining cell nuclei, we observed a marked decrease in GFP expression when driven by the CpG containing CAG promoter; however, GFP expression driven by CpG-free EF1a was not significantly altered by methylation. These preliminary results demonstrate that methylation-dependent silencing of transgene expression can be addressed by CpG-free promoters. To address scalability, the MT sequence was codon optimized for *E. coli* and non-canonical stop codons were removed. MT was integrated into the *E.coli* genome under control of an inducible Tet promoter using a Tn7 transposase downstream of the bacterial glmS gene locus and verified by sequencing. The MT expressing *E. coli* was transformed with vector plasmid pAAV2-CpGfreeEf1a>GFP and upon expansion and induction provided high yield of the vector plasmid that after purification and transfection expressed GFP in cell culture. Digestion with methylation insensitive vs sensitive restriction enzymes confirmed plasmid DNA methylation. Parallel studies to integrate MT into HEK293 genomes by lentiviral transduction were also performed yielding MT positive cells that are currently being characterized. These results support the feasibility of biosynthetic approaches to generate recombinant AAV vectors with increased CpG methylation, providing a novel vector and process approach to address the challenge of TLR9-associated innate immune activation caused by the inherent hypomethylation of AAV vector genomes.

212 B-cell Targeted Therapies Deplete Neutralizing Antibodies to AAV

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Background: A major barrier to adeno-associated viral (AAV) vector gene therapy is the presence of persistent, multi-serotype cross-reactive neutralizing antibodies (NAbs) that develop following wildtype AAV infection and AAV vector infusion that render patients ineligible for initial or repeat AAV dosing. Prior human studies have evaluated plasmapheresis and various immunomodulatory agents to remove NAbs to allow therapeutic vector administration. These studies have been limited by an incomplete evaluation of the B-cell compartments essential to AAV NAb persistence. Understanding the anti-AAV B-cell response would aid in the development of rational strategies to reduce or eliminate AAV NAbs. Here, we aim to systematically investigate the ability of B-cell immunomodulation strategies, including engineered T-cell immunotherapy, to deplete pre-existing AAV NAbs and facilitate AAV gene therapy. **Methods:** AAV NAb titers were measured via a transduction inhibition assay using an AAV8 luciferase reporter vector. Wild type C57Bl/6 mice were immunized with an AAV8 vector at 1 x 10^{11} vg/mouse to establish AAV NAbs. Two weeks later, animals were administered chimeric antigen receptor (CAR) T-cells that were either non-transduced (NTD, controls) or targeted mouse CD19, mouse B-cell maturation antigen (BCMA), or both (Fig 1A). Elevent weeks after CAR-T infusion, mice were re-challenged with an AVV8 vector carrying factor VIII (FVIII) at 1 x 10^{11} vg/mouse. Plasma FVIII antigen levels were measured by ELISA to assess transduction capacity. **Results:** Initial AAV exposure resulted in NAb titers prior to CAR-T therapy that were not different between experiment groups (median 1:336, range 1:95 - 1:1653, Fig 1B). Significant and persistent B-cell aplasia was noted after CD19 (12.4 ± 10.6 cells/μL) and CD19+BCMA (1.8 ± 3.5) CAR-T infusion in contrast to NTD or BCMA alone CAR-T infusion (129 ± 80 and 106 ± 80, respectively, $p < 0.05$). Following CAR-T infusion, NAb titers in the NTD and BCMA-only CAR-T groups continued to rise to greater than 1:3200 whereas mice treated with CD19-targeting CAR-T regimens had mean titers below 1:5 (Fig 1B, $p < 0.05$). There was no difference between the CD19-only and CD19+BCMA regimens. Upon AAV8-FVIII re-challenge, FVII antigen levels inversely correlated with NAb titer (Spearman r -0.47, $p < 0.05$) with CD19-targeting CAR-T regimens resulting in significantly higher expression of FVIII antigen compared to NTD or BCMA CAR-T regimens (19.9 ± 28.1 vs 5.6 ± 3.8 ng/ml, respectively, $p < 0.05$). **Conclusions:** These data support that CD19-targeted therapy can sufficiently reduce AAV NAb titers to below 1:5 and enable AAV vector transduction in pre-exposed mouse models. Additionally, the data support that a CD19+ B-cell population is important in the ongoing AAV NAb response in mice. The lack of difference in NAb titers between the NTD and BCMA-only CAR-T groups or between CD19-only or CD19+BCMA CAR-T groups suggests that CD19-BCMA+ long-lived plasma cells are not the primary cellular reservoir of anti-AAV humoral immunity. However, given the longevity of the AAV NAb response in humans, ongoing studies of samples from patients treated with B-cell targeting biologics and CAR-T regimens will inform the significance of the plasma cell compartment in AAV immunity in humans.

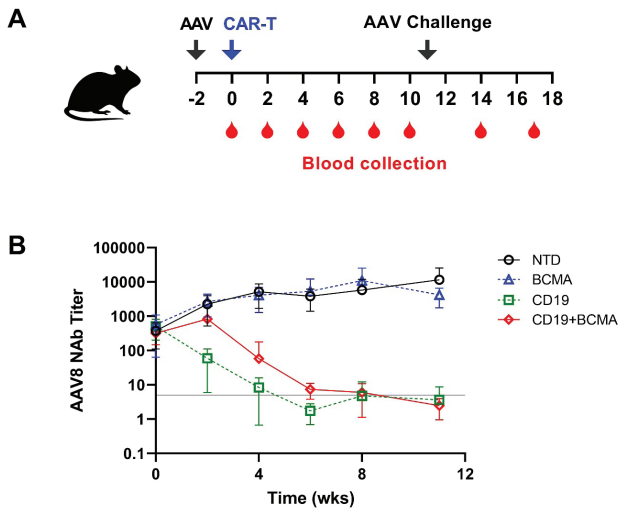

Figure 1. A) Experimental schema. **B)** AAV8 NAb titer after CAR-T injection.

213 Redosing of AAV Vectors Containing TLR9 Inhibitory Oligonucleotides Improves Survival in a Mouse Model of Respiratory Surfactant Deficiency

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Surfactant protein B (SP-B) deficiency (OMIM#265120) is a lethal genetic lung disease that impairs pulmonary surfactant production, with affected individuals dying of respiratory distress three to six months after birth. We previously identified a genetically engineered adeno-associated viral (AAV6.2FF) vector (AAV-hSPB) capable of transducing alveolar epithelium and improving lung structure, physiology, and survival in a mouse model of SP-B deficiency. Although median survival improved to **140 days** compared to **2-7 days** without gene therapy, the effects of AAV-hSPB waned, and the mice eventually died of respiratory distress. A potential reason for this loss in vector efficacy is the immune response (IR) to AAV. The toll-like-receptor 9 (TLR9) pathway plays a critical role in the initial IR against AAV, and strategies have been developed to suppress TLR9 activation in the hopes of improving gene therapy efficacy. One strategy is the incorporation of oligonucleotide sequences (TTAGGG) into the AAV vector genome to inhibit the TLR9 pathway, which has shown promise in prolonging transgene expression and suppressing the IR in the liver, muscles, and retina. To test the potential effects of TLR9 inhibition in the lungs, we inserted TLR9 inhibitory oligonucleotide (io) sequences into AAV-hSPB (AAV-hSPB-io2). A single dose of AAV-hSPB-io2 significantly improved median survival to **205 days** compared to only **134 days** in mice administered a single dose of AAV-hSPB (P=0.0043). The eventual decrease in survival could be overcome if AAV-hSPB could be redosed. However, the adaptive IR that develops after an initial dose prevents efficient AAV readministration. The suppression of the IR by TLR9 inhibition led us to propose that redosing of gene therapy vectors into the lungs is possible using this strategy. Readministration of AAV-hSPB resulted in a median survival of **122 days**. In sharp contrast, repeated dosing of AAV-hSPB-io2 every 90 days improved median survival to **296 days** (P<0.0001), with the longest surviving mouse living for **357 days**. Treatment with AAV-hSPB-io2 results in no adverse effects in mice as observed by steady increases in body weight and the lack of an inflammatory cytokine profile. Gene expression analyses reveals that redosing with AAV-hSPB significantly alters the expression of 136 out of more than 750 genes that were measured from the murine immune and inflammatory response pathways. This includes several genes (*Cxcl9*, *Cxcl10*, *Tap1*, *Stat1*, *Psmb9*, and *Isg15*) from the type II interferon pathway (adjusted P=0.038). However, the changes in gene expression are suppressed when mice are readministered with AAV-hSPB-io2. Only 9 genes demonstrate significant changes out of the same 750 genes measured. TLR9 inhibitory sequences also impedes the expression of gene markers associated with T helper cells, but does not affect expression of B cell markers or serum antibody levels against AAV. These findings demonstrate that TLR9 inhibitory sequences allow for AAV readministration in the lungs through the broad disruption of multiple but not all components of the IR against AAV.

367 Establishing Multilayered Genetic Resistance to HIV-1 by Engineering Hematopoietic Stem and Progenitor Cells for B Cell Specific Secretion of Therapeutic Antibodies

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Autologous transplantation of CCR5 null hematopoietic stem and progenitor cells (HSPCs) is the only known cure for HIV-1 infection. However, this treatment is extremely limited due to the rarity of CCR5 null matched donors, the morbidities associated with allogeneic transplantation, and the prevalence of HIV-1 strains resistant to CCR5 knockout (KO) alone. Here, we propose a one-time therapy through autologous transplantation of HSPCs genetically engineered *ex vivo* to deliver both CCR5 null cells and long-term secretion of potent anti-HIV-1 antibodies. We employ highly efficient CRISPR-Cas9/AAV6 mediated genome editing techniques to simultaneously knock-out CCR5 and knock-in anti-HIV-1 antibody expression cassettes for multi-layered cell autonomous and cell non-autonomous HIV-1 resistance, respectively. We achieve over 90% knock-out efficiency and 25-35% knock-in frequency at the *CCR5* locus in primary HSPCs. We utilize a previously defined B cell specific promoter to drive expression of Ibalizumab and 10-1074, highly potent anti-HIV-1 antibodies used in combination to limit viral escape and provide broad resistance across viral strains. We engineer these antibodies for expression with a peptide linker physically pairing the light and heavy chains to prevent mispairing with endogenously produced antibodies. We demonstrated our engineered antibodies maintain their efficacy compared to their unmodified counterparts in an HIV-1 neutralization assay. Further, we achieved efficient targeted integration of our antibody expression cassettes in primary human B cells with frequencies up to 43% in the *CCR5* locus. We show that B cells edited to express 10-1074, Ibalizumab, or both antibodies secrete neutralizing levels of antibody in culture supernatant. After validating our system, we engineered HSPCs with CCR5 KO and integration of the 10-1074 antibody cassette for engraftment in sublethally irradiated NSG mice. Endpoint analysis at week 13 showed human chimerism up to 65%, and human cells containing our integrated antibody cassette were maintained, demonstrating persistence of our engineered cells *in vivo*. Importantly, serum taken from the engrafted mice generated highly efficient neutralization of HIV-1 pseudovirus when applied to an HIV-1 infection assay. Serum antibody concentrations estimated from the neutralization efficiency range from 40 μg/mL to 290 μg/mL, greatly surpassing the clinically relevant concentration of 10 μg/mL. Ongoing studies combine expression of multiple antibodies to deliver resistance to broad strains of HIV-1 and prevent the development of viral resistance. Here, we establish a highly efficient system to

simultaneously knock-out CCR5 and knock-in B cell specific antibody expression cassettes in HSPCs to engineer multilayered resistance to HIV-1. We believe this strategy has the potential to become a one-time therapy that leads to long-term control of HIV-1 infection, a disease that currently requires lifetime administration of anti-retroviral therapy to maintain viral suppression.

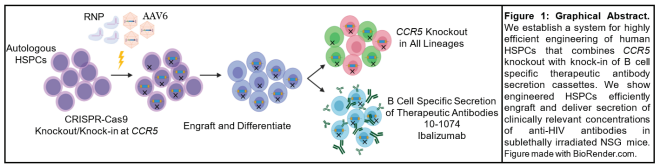

Figure 1: Graphical Abstract. We establish a system for highly efficient engineering of human HSPCs that combines *CCR5* knockout with knock-in of B cell specific therapeutic antibody secretion cassettes. We show engineered HSPCs efficiently engraft and deliver secretion of clinically relevant concentrations of anti-HIV antibodies in sublethally irradiated NSG mice. Figure made with BioRender.com.

Hematologic and Immunologic Diseases

214 mRNA Delivery to Hematopoietic Stem Cells by Targeted Lipid Nanoparticles Allows *In Vivo* Genome Engineering and Control of Cell Fate

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Disorders of the blood, such as hemoglobinopathies and immunodeficiencies, can be cured by correcting or replacing diseased hematopoietic stem cells (HSC). Gene therapy (GT) for these non-malignant hematopoietic disorders (NMHD) is currently done with *ex vivo* lentiviral transduction of HSC or electroporation with genome editors. Myeloablative conditioning is required for HSC transplant (HSCT) of autologous gene-modified HSC. Conditioning is based on chemotherapy or radiation and has acute and chronic morbidity and mortality. We developed a novel CD117 (stem cell factor receptor) targeted lipid nanoparticle encapsulating modified mRNA to achieve *ex vivo* and *in vivo* genome engineering and control of cell fate. To evaluate LNP targeting, murine cre-responsive reporter models (Ai6, Ai9, Ai14) were utilized with LNP encapsulating Cre mRNA (LNP-Cre). Anti-CD117 targeted LNP-Cre (CD117/LNP-Cre) produced 95% genome editing in LSK cells after 6 hours treatment. To evaluate the multipotency of targeted cells, we transplanted C57BL/6 CD45.1 recipient mice with Ai14 BM cells treated *ex vivo* with increasing doses of CD117/LNP-Cre and Control IgG/LNP-Cre. 95% of long-term HSC (LT-HSC, $Lin^-Sca1^+cKit^+CD150^+CD48^-$) had been targeted at four months post-transplant (Fig. 1A), with all blood lineages showing edited cells (Fig. 1B) These HSC were found to be self-renewing utilizing secondary transplantation. We administered CD117/LNP encapsulating luciferase mRNA intravenously (IV) and observed activity in the bone marrow (Fig. 1C). IV administration of CD117/LNP-Cre (5μg) in Ai9 mice yielded 55% LT-HSC targeting at 4 months post-treatment, 2.9-fold higher than seen with Control IgG/LNP-Cre (Fig. 1D). Transplantation of BM from these animals phenocopied the donor bone marrow. Targeting of LNP to human CD117 (hCD117/LNP) encapsulating cas9-adenine base editor (NRCH-cas9-ABE8e) mRNA and gRNA converted 87% of alleles from the sickle hemoglobin mutation (Hbb^S) to the G-Makassar variant (Hbb^G) in erythroblasts derived from sickle cell disease affected $CD34^+$ cells *in vitro* (Fig. 1E). Hbb^G was 90% of β-like globins (Hbb^G+Hbb^S) in edited cells after erythroid differentiation (Fig. 1F). Hypoxia induced sickling in edited SCD erythroblasts was reduced by 96% compared to control cells (Fig. 1G and 1H). *In vivo* targeting of HSC offers the ability to modify HSC fate, potentially a therapeutic modality for NMHD. We tested the ability of pro-apoptotic mRNA as non-genotoxic conditioning for HSCT. mRNA expression in the liver was reduced by adding a miRNA binding site. CD117/LNP-PUMA induced HSC depletion *in vivo* and allowed for engraftment of GFP^+ donor BM. At the maximum tolerated dose, 3.7% of LSK cells were donor derived after four months, which is a therapeutic level of engraftment in some immunodeficiencies (Fig. 1I). These results show that LNP mRNA delivery can be controlled both by targeting and manipulation of the mRNA message. This may revolutionize the treatment of genetic diseases in two ways. First, the cure of NMHD disorders, such as sickle cell disease may be attainable with a simple infusion that corrects the causative molecular defect. Second, effecting cell-type specific state changes *in vivo* will allow previously impossible manipulations of physiology.

Figure 1.

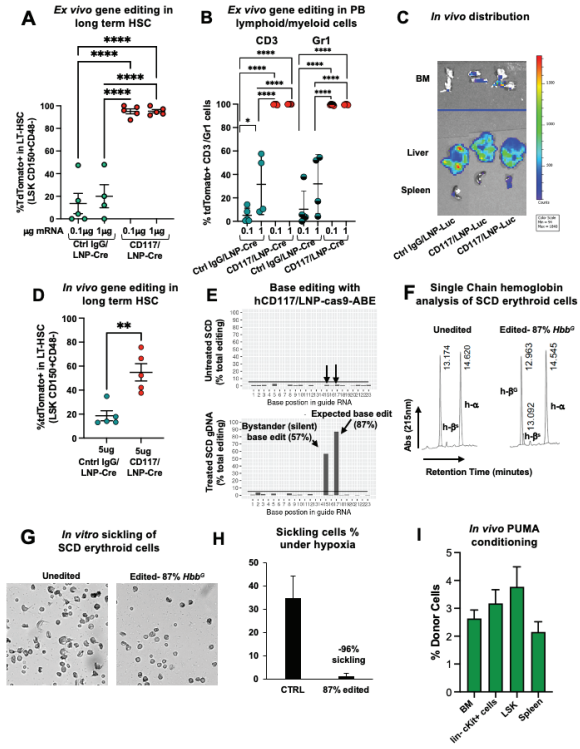

Figure 1. (A) Percentage of reporter positive bone marrow long-term HSC (LSK $CD150^+CD48^-$) and peripheral blood (PB) (B) CD3+ (lymphoid) and Gr1+ (myeloid) cells 4 months post-transplant in lethally irradiated congenic recipients engrafted with BM of Ai14 (tdTomato reporter) mice treated with IgG/LNP-cre or CD117/LNP-Cre *ex vivo* for 18 hours. (C) Luciferase activity in bone marrow, liver, and spleen after CD117/LNP-luciferase administered I.V. (D) *In vivo* editing in LT-HSC cells 4 months after I.V. injection of LNP-Cre. (E) Mutation analysis of sickle cell disease (SCD) erythroblasts after treatment with human (h) CD117 targeted LNP carrying cas9-adenine base editor (ABE) mRNA and separately hCD117/LNP carrying gRNA. (F) Reverse-phase HPLC analysis of single globin chains from unedited and hCD117/LNP-cas9-ABE + gRNA treated SCD erythroblasts. (G) Representative images from unedited and edited SCD erythroblasts exposed to hypoxic conditions and (H) quantification of the frequency of sickled cells by morphology (N=5 high powered fields per condition). (I) Engraftment analysis in bone marrow subsets after 4 months of mice receiving CD117/LNP-PUMA as conditioning and donor GFP^+ BM cells.

215 First Case of Leukaemia in Retroviral Gene Therapy for ADA-SCID

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Hematopoietic stem cell (HSC) gene therapy (GT) using a γ-retroviral vector (γRV) is an effective treatment for Severe Combined Immunodeficiency due to Adenosine Deaminase deficiency (ADA-SCID). Here, we describe the first occurrence of drug-related T-cell acute lymphoblastic leukemia (T-ALL) that developed 4.7 years after GT. Routine follow-up until 3 months before the event onset did not reveal any immunological or clinical abnormalities. After diagnosis, the patient underwent chemotherapy and, after remission, HLA-haploidentical transplantation and is in molecular remission up to 20 months post-transplant. Blast cells contained a single vector activating insertion near the LMO2 proto-oncogene, confirmed by direct physical interaction, and showed low ADA activity resulting from methylation of viral promoter sequences. Mature T cells and other blood lineages harboring the insertion at the LMO2 locus were detected at stable levels years before T-ALL onset, suggesting that further mutagenic hits occurred at the level of a thymic progenitor. Indeed, a variety of known and novel somatic mutations including intragenic and structural rearrangements possibly contributed to orchestrate the development of the T-ALL. Interestingly, a clone with an activating insertion in the MECOM gene increased to 25% in myeloid cells during chemotherapy treatment for T-ALL and disappeared after allogeneic transplantation. It is possible that MECOM overexpression conferred a fitness advantage to this clone favoring its expansion and the development of clonal hematopoiesis during chemotherapy. Before T-ALL onset, the overall insertion profile and common insertion sites were similar to those of other γRV-treated ADA-SCID patients using the same vector, without the presence of prominent clones in proto-oncogenes. As compared to another ADA-SCID clinical trial based on γRV with a different viral promoter, the only major difference was the targeting frequency of MECOM which was significantly lower in our trial. The limited frequency of vector-related adverse events in γRV ADA-SCID GT, as compared to other immune deficiencies, suggests a predisposing role of disease and possible predisposing factors in this patient. While γRV GT for ADA-SCID maintains a positive benefit-risk ratio, the identified risk of insertional oncogenesis requires long-term safety monitoring of treated patients.

216 Lentiviral Vector Mediated *In Vivo* Gene Transfer into Hematopoietic Stem and Progenitor Cells

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In the last decade, lentiviral vector (LV) mediated *ex vivo* gene therapy in hematopoietic stem and progenitor cells (HSPC) fulfilled the promise of a long-term treatment for a number of genetic diseases, including primary immunodeficiencies and hematological disorders. However, collection of high number of cells, their *ex vivo* manipulation and conditioning of the patient still pose challenges to broad access to HSPC gene therapy. To investigate the feasibility of *in vivo* LV-mediated gene transfer into HSPC, we systemically administered GFP-expressing LV to adult or newborn mice and found only in the latter stable GFP expression in 1% of all blood lineages up to 1-year. We observed comparable GFP marking in hematopoietic organs and in HSPC harvested from the bone marrow (BM), which were able to engraft long-term in busulfan-conditioned recipient mice. LV integration site analysis confirmed common origin of different hematopoietic lineages from multiple clones. These findings indicate successful *in vivo* gene transfer into *bona fide* HSC favored by some unique features of newborn hematopoiesis, such as access to hepatic hematopoietic niches persisting after birth, and HSC-trafficking to the BM. In order to evaluate newborn HSPC potential, we isolated hematopoietic cells from the liver or the BM of newborn mice and transplanted them in conditioned adult recipient mice. We observed similar engraftment and hematopoietic output in mice transplanted with cells isolated from the two sources and this was confirmed even upon secondary transplant. To increase gene transfer efficiency we exploited phagocytosis-shielded CD47-high LV and showed 3-fold higher gene marking in the blood and BM of treated mice. Sorted lineage-negative cells from the BM generated GFP-positive colonies *ex vivo*. Biodistribution studies showed transgene-positive cells in other relevant organs, such as the liver, the spleen, the brain, the lungs and the kidneys of newborn-treated mice, comprising hematopoietic, endothelial and in some cases parenchymal cells. While expression in non-hematopoietic cells could be avoided by tailored vector expression, it might be advantageous for treating systemic diseases. As a model for systemic disease correction, we administered LV expressing the adenosine deaminase (ADA) enzyme to ADA-SCID newborn mice and showed rescue from lethal phenotype and reconstitution of lymphocyte counts with selective advantage of LV-transduced cells. Twenty weeks after LV administration, we transplanted BM cells from ADA-SCID mice treated as newborns into NSG recipient mice and

showed long-term LV marking of around 5% in the BM and lymphoid organs of transplanted mice, in line with marking observed in donor mice treated as newborns. Our work shows *in vivo* gene transfer into HSPC in newborn mice and life-long maintenance of transgene expression, suggesting that special hematopoietic niches and higher HSPC accessibility in this window may be exploited to obtain disease correction, especially when a selective advantage of corrected cells is present. Further studies are ongoing to explore *in vivo* LV gene transfer into HSPC in other disease models.

217 Lentiviral-Mediated Gene Therapy for Fanconi Anemia [Group A]: Results from Global RP-L102 Clinical Trials

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Background: Fanconi anemia (FA) is an inherited deoxyribonucleic acid (DNA) repair disorder that results in progressive bone marrow failure (BMF) in 80% of patients within the first decade of life. Allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative for FA-related BMF. Although survival exceeds 80% in experienced transplant centers, adverse effects including 100-day mortality and increased cancer risk present persistent limitations. The current studies utilize autologous FA-A CD34+ enriched hematopoietic stem and progenitor cells (HSPCs) and rely upon the proliferative advantage of gene-corrected FA HSPCs, enabling engraftment without conditioning. We report results from global RP-L102 studies. **Methods:** Patients with *FANCA* mutations, age ≥1 year with no HLA-matched sibling donor and ≥30 CD34+ cells/μL in bone marrow (BM) are eligible. Peripheral blood (PB) cells are collected via leukapheresis. Following CD34+ enrichment, HSPCs are transduced with a lentiviral vector carrying the *FANCA* gene, and infused without cryopreservation or conditioning. Patients are followed for 3 years for safety assessments (including insertion site analysis [ISA]) and for evidence of efficacy (increasing vector copy number [VCN], mitomycin-C [MMC] resistance in BM colony forming cells [CFCs]), and stabilization/correction of cytopenias). **Results:** As of October 2022, 12 patients age 2 to 6 years have received RP-L102. Sustained engraftment has been demonstrated in 7 of 10 evaluable patients with ≥12 months of follow up as indicated by peripheral blood mononuclear cell (PBMC) VCN (median: 0.53; range: 0.19-0.65 at 12-40 months). Six of these 7 patients have increasing BM CFC MMC resistance with concurrent hematologic stabilization (median: 85%; range: 22-94% at 12-40 months). One patient without genetic correction had progressive BMF and underwent successful alloHSCT. A transient serious Grade 2 RP-L102 infusion-related reaction was observed in one patient and resolved without sequelae. No patients have developed RCL. One patient developed T cell lymphoblastic lymphoma determined to be unrelated to gene therapy. There has been no evidence of RP-L102 related bone marrow dysplasia, clonal dominance or insertional mutagenesis. **Conclusions:** RP-L102 conferred phenotypic correction as demonstrated by sustained increase in BM CFC MMC resistance, genetic correction and hematologic stabilization in at least 6 patients with ≥1 year of follow up. Sustained engraftment, phenotypic correction, and hematologic stability was achieved in the absence of conditioning. RP-L102 represents a potentially curative therapy for FA-related BMF, which can be administered without transplant-conditioning related toxicities. Updated results for patients (n=12) with ≥ 1 year of follow-up will be presented.

218 Global Phase 1 Study Results of Lentiviral Mediated Gene Therapy for Severe Pyruvate Kinase Deficiency

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Background: Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia caused by *PKLR* gene mutations. Manifestations include anemia, splenomegaly and iron overload, which may be life-threatening. Currently available treatments are limited to a recently-approved enzyme activator or palliative therapies such as chronic blood transfusions, iron chelation therapy and splenectomy which are associated with significant side effects. A global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate lentiviral mediated hematopoietic stem and progenitor cell (HSPC)-targeted gene therapy for adults and children with severe PKD. **Methods:** Splenectomized patients with severe PKD (severe and/or transfusion-dependent anemia) are eligible. Following apheresis, HSPCs are transduced with lentiviral vector and cryopreserved. Myeloablative therapeutic drug monitoring-guided busulfan is administered and the gene therapy product (RP-L301) is thawed and infused. Patients are followed for safety assessments (including insertion site analysis [ISA]), and efficacy (genetic correction, decrease in transfusion requirements, significant improvement in anemia and reduction of hemolysis) for 2 years post-infusion. **Results:** As of October 2022, 2 patients (age 31 and 47 years at enrollment) with severe anemia have received RP-L301. Patient 1 received $3.9x10^6$ CD34+ cells/kg with mean vector copy number (VCN) of 2.73. Patient 2 received $2.4x10^6$ CD34+ cells/kg with mean VCN of 2.08. Despite baseline hemoglobin (Hb) levels in the 7.0-7.5 g/dL

range, at 24 months post-infusion both patients have normal-range hemoglobin (13.2 g/dL and 14.7 g/dL, respectively), and no red blood cell transfusion requirements post-engraftment. Other parameters of hemolysis and anemia (LDH, bilirubin, erythropoietin) are improved. Peripheral blood mononuclear cell (PBMC) vector copy numbers (VCNs) were 1.75 and 1.43 at 24-months. Both patients reported improved quality of life (QOL), also demonstrated by increases in both FACT-An and SF-36 scores, with marked improvement in SF-36 energy/fatigue, physical functioning, and general health components. No serious adverse events (SAEs) have been attributed to RP-L301. Hematopoietic reconstitution occurred within 2 weeks of administration. ISA in PB and BM for both patients up to 12 months following therapy indicate highly polyclonal patterns; longitudinal results delineating clonal diversity will be presented. **Conclusion:** Clinical efficacy and safety data indicate that RP-L301 is a potential treatment for patients with severe PKD, including those who did not derive benefit from available therapies. Robust and sustained efficacy in both patients at 24 months post-treatment was demonstrated by normalized hemoglobin, improved hemolysis parameters, and transfusion independence.

219 Unraveling the Effect of Proliferative Stress *In Vivo* in Hematopoietic Stem Cell Gene Therapy Mouse Study

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The hematopoietic system of patients enrolled in hematopoietic stem cells (HSC) gene therapy (GT) treatments is fully reconstituted upon autologous transplantation of engineered stem cells. HSCs highly proliferate up to full restoration of homeostasis and compete for niche homing and engraftment. The impact of the proliferation stress in HSC on genetic instability remains an open question that cured patients advocate for characterizing long-term safety and efficacy. The accumulation of somatic mutations has been widely used as a sensor of proliferative stress. Vector integration site (IS) can be used as a molecular tool for clonal identity, inherited by all HSC progeny, to uncover lineage dynamics in vivo at single-cell level. Here we characterized at single-clone granularity the proliferative stress of HSCs and their progeny over time by measuring the accumulation of mutations from the DNA of each IS. To test the feasibility of the approach, we set-up an experimental framework that combines tumor-prone *Cdkn2a*$^{-/-}$ and wild type (WT) mouse models of HSC-GT and molecular analyses on different hematopoietic cell lineages after transplantation of HSCs transduced with genotoxic LV (LV.SF.LTR) or GT-like non-genotoxic LV (SIN.LV.PGK). The *Cdkn2a*$^{-/-}$ mouse model provided the experimental conditions to detect the accumulation of somatic mutations, since the absence of p16^{INK4A} and p19^{ARF} enhances the proliferative potential of cells that have acquired oncogenic mutations. As expected, mice transplanted with *Cdkn2a*$^{-/-}$ Lin^{-} cells marked with LV.SF.LTR (N=24) developed tumors significantly earlier compared to mock (N=20, $p<0.0001$), while mice treated with SIN.LV.PGK (N=23) did not. On the other side, mice that received WT Lin^{-} cells treated with LV.SF.LTR (N=25) or SIN.LV.PGK (N=24) vector have not developed tumors. Given this scenario, we expect that *Cdkn2a*$^{-/-}$ Lin^{-} cells transduced with LV.SF.LTR are associated with higher mutation rates compared to the SIN.LV.PGK group and wild type control mice. The composition of peripheral blood, lymphoid (B and T) and myeloid compartments was assessed by FACS on samples collected every 4 weeks and IS identification. More than 200,000 IS have been recovered. To identify the presence of somatic mutations, the genomic portions of sequencing reads flanking each different IS were analyzed with VarScan2. The accumulation rates of mutations have been evaluated by our new Mutation Index (MI) which normalizes the number of mutations by clones and coverage. Considering that a large portion of IS has been discarded since not covered by a minimum number of 5 unique reads (genomes), the remaining number of IS contained >90% of reads in each group. The MI increased over time in both LV.SF.LTR groups, with higher values for the *Cdkn2a*$^{-/-}$. On the other hand, treatment with SIN.LV.PGK resulted in lower MI in both groups compared to LV.SF.LTR groups, reflecting the higher clonal composition of the cells treated with the SIN.LV.PGK and the phenomenon of insertional mutagenesis in the LV.SF.LTR. Moreover, the higher MI values of the SIN.LV.PGK *Cdkn2a*$^{-/-}$ group compared with the WT group proved the induction of DNA fragility. Our results showed that the analysis of the accumulation of somatic mutations at single clone unraveled HSC proliferation stress in vivo, combining for the first time the analysis of acquired mutations with IS. We are now applying our model to different clinical trials, and studying HSCs sub-clonal trees by symmetric divisions, previously indistinguishable by IS only. Our study will open the doors to in vivo long-term non-invasive studies of HSC stability in patients.

220 Highly Efficient Correction of the Sickle Cell Disease Mutation in Patient HSC Using an RNA Gene Writing System, an RNA-Based, Nuclease-Free Approach to Genome Editing

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The ability to introduce new DNA sequences into the genome with high specificity and efficiency would provide a critical complement to existing gene editing approaches. Here, we developed Gene Writing systems, leveraging target-primed reverse transcription (TPRT) biochemistry evolved by non-LTR retrotransposon mobile genetic elements to edit genetic information at a specific genomic site without introducing DNA breaks in hematopoietic stem cells (HSC). Programmable TPRT requires the enzymatic steps of DNA nicking and reverse transcription. Using a large throughput screening, we developed synthetic Gene Writer enzymes that can catalyze a variety of editing reactions, such as the introduction of gene-length DNA sequence and substitutions, including the correction of *HBB* sickle mutation

to WT. These edits can be achieved with all-RNA delivery in primary cells and in vivo, eliminating the necessity for viral vectors and DNA template-based gene editing. Sickle cell disease (SCD) is caused by a point mutation in the *HBB* gene (E6V), affects erythroid lineage leading to severe anemia and significant multiorgan complications. Challenges of current gene therapy approaches for SCD include safety concerns due to insertional oncogenesis risk by viral vectors, DNA double-strand break induction by nucleases, and inadequate achievement of corrective levels of HSC modification. Using our Gene Writer system comprised of a reverse transcriptase fused to a synthetic RNA-guided Cas nickase and a modified template RNA, we first modeled the efficiency of *HBB* gene modification by introducing the *HBB* Makassar variant (E6A) in CD34+ human HSC from normal donors. Extensive studies optimizing the RNA template and delivery/expression of our Gene Writer system showed we could achieve up to 70% perfect allele modification. Similar editing efficiency was observed in quiescent HSC as compared to cytokine-stimulated cells, results that are consistent with our platform not requiring cell division or engagement by the HDR machinery. As compared to treatment with conventional nucleases, our Gene Writing system did not induce a detectable p53-related response. We then validated efficient editing of long-term repopulating HSC by demonstrating similar editing efficiencies in sorted CD90+ primitive cells and achieving ~70% perfect HBB modification in HSC and their multi-lineage progeny isolated from transplanted mice. No phenotypic or functional impairment was observed in edited HSCs as measured by colony forming assays, erythroid differentiation, or in vivo reconstitution studies up to 16 weeks post-transplant. Finally, we achieved efficient ex vivo editing of CD34+ cells from SCD patients, showing ~70% correction of sickle alleles to WT. Differentiation towards erythroid lineage showed gene modified cells exhibited improved enucleation and, importantly, restoration of normal adult hemoglobin levels by HPLC (98% HbA, 2% HbS) and LC-MS. Our editing strategy enabled phenotypical correction of virtually all differentiated erythrocytes, resulting in complete abrogation of cell sickling. Given that the all-RNA nature of our compositions potentially enables in vivo strategies for correcting SCD, we also showed we are able to efficiently deliver mRNA to HSC in animal models by LNP (>80% in humanized mice, >30% in NHP). These results highlight that our Gene Writing technology based on TPRT biochemistry is highly efficient at introducing precise edits in human HSC and provides a promising new therapeutic approach to SCD.

Cell Therapy Product Engineering and Development | Cancer

221 Allogeneic CAR T Cells with Regulated NEF Expression Display Immune Evasion and Optimized Signaling

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Allogeneic CAR T cells manufactured from healthy donor or pluripotent stem cells are a potential off-the-shelf alternative to current autologous therapies. Allogeneic products could reduce cost and allow rapid treatment, but are limited by host-versus-graft immune (HVGI) rejection. To study HGVI, we developed a leukemia xenograft model in immunodeficient mice engrafted with PBMC, and demonstrated that CD8 T cell responses limit allogeneic CAR T cell persistence and tumor control. To develop an allogeneic CAR T cell resistant to immune rejection, we used retroviral vectors to co-transduce CAR and a panel of viral immune evasion proteins, including US2 and US11 from hCMV, K5 from KSV, BNLF2a from EBV, and NEF from HIV1. All evasion proteins downregulated MHC-I surface expression to varying degrees, leading to resistance from CD8 T cell killing *in vitro*. However, variegated expression from a retroviral promoter led to variable and transient downmodulation of MHC levels *in vivo*. In contrast, site-specific genomic insertion from a constitutive promoter leads to stable expression, durable MHC downregulation, and longer in vivo persistence. We next utilized a tumor-only xenograft model to show that expression of HIV-1 NEF but not other evasion proteins intrinsically enhanced CAR T cell expansion and tumor control. Using phosphoproteomics, we demonstrated that that NEF attenuates CAR T cell signaling, including decreased CD3ζ and CD28 phosphorylation. We further demonstrated this function depends on the NEF SH3-binding domain that modulates T cell signaling through interactions with several proteins, including LCK. As a result, regulated NEF expression led to stable MHC downregulation, increased T cell expansion, and improved tumor clearance in a PBMC-engrafted NALM6 leukemia model and a CD19 expressing glioblastoma model. Thus, we achieve a clinically translational allogeneic CAR T cell platform by enhancing both intrinsic CAR T cell function and immune evasion.

222 Mechanistic Characterization of Afamitresgene Autoleucel (Afami-cel; Formerly ADP-A2M4)

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Afami-cel is a T-cell receptor (TCR) T-cell therapy composed of autologous CD4+ and CD8+ T-cells genetically modified to express an engineered TCR targeting the cancer testis antigen MAGE-A4. Afami-cel demonstrated encouraging results and an acceptable benefit-to-risk profile in adults with relapsed/refractory metastatic

solid cancers, especially synovial sarcoma (SyS). In the Phase 1 trial of afami-cel (NCT03132922), there was an overall response rate (ORR) of 44%, a disease control rate of 94%, and a median duration of response of 6.4 months in patients with SyS.[1] Results from Cohort 1 of the Phase 2 trial of afami-cel (SPEARHEAD-1; NCT04044768) show an ORR of 38.6% and durable responses (median of 11.6 months) in SyS.[2] Ongoing translational analyses evaluate if there is optimal phenotypic composition of afami-cel manufactured product (MP), conferring better tumor killing response, proliferative capacity, and ability to survive in the tumor. Here we examine how afami-cel MP phenotypic composition translates to functional profile. MP was sampled before infusion into patients in SPEARHEAD-1. Samples were stimulated using magnetic particles labelled with MAGE-A4-specific peptide-HLA and anti-CD28 antibodies, with or without interleukin 2. Unstimulated and stimulated MP were phenotypically profiled by flow cytometry using multi-color staining panels for T-cell exhaustion and activation at Day 0 and after 24 h. Functional cytotoxicity and proliferation profiling was performed on unsorted and FACS-sorted T-cell subpopulations. Cytotoxicity assays using Incucyte imaging examined tumor derived cell line growth (A375nucGFP$^+$) in the presence or absence of MP. T-cell proliferation and death were measured simultaneously over 6 d using RealTime Glo MT cell viability and CellTox Green cytotoxicity assays. Mitochondrial metabolic state was determined by flow cytometry using MitoTracker Green FM and Red CMXRos to quantify mitochondrial mass and membrane potential at Day 0 and 24 h after stimulation. Preliminary mechanistic analyses were performed for three MP samples, which showed a variable range in manufactured dose (4.7-9.4 $\times 10^9$ cells), transduction efficiency (25-77% transduced cells), and CD4+ (32-52%) and CD8+ (44-66%) T-cell frequencies. Flow cytometry profiles showed differential stimulation-induced increases in (co-)expression levels of activation/exhaustion markers within sample subpopulations and between samples, related to functional responsiveness. Overall, the MP sample with the highest manufactured dose and percentage of transduced cells showed the greatest cytotoxic response and proliferative capacity, as indicated by faster kinetics and magnitude of CD4+/CD8+ T-cell proliferation in response to stimulation. The converse was noted for the MP sample with the lowest manufactured dose and percentage of transduced cells; the lower proliferative capacity was shown to not result from increased cell death. The MP with lowest proliferative and cytotoxic capacity showed a higher mitochondrial mass at baseline and a lack of mitochondrial response post stimulation. Initial data demonstrate proof-of-concept translation of afami-cel MP phenotype to in vitro function and indicate a relationship to manufacturing dose and transduction efficiency. To understand if the mechanistic capacity of MP indicates postinfusion clinical response, expanded MP datasets with SPEARHEAD-1 clinical correlatives will be presented.1. Hong, et al. Nat Med (2023). https://doi.org/10.1038/s41591-022-02128-z. 2. Van Tine, et al. Paper 61: CTOS 2022; Vancouver, Canada

223 Cytosine Base Editor Ameliorates the Safety Profile of (TCR) - Transgenic T Cells for the Adoptive Cell Therapy of Gastrointestinal Tumors

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Adoptive cell therapy (ACT) with T cell receptor (TCR) - transgenic T cells is a promising therapeutic strategy for advanced gastrointestinal cancers. However, the paucity of TCRs targeting relevant tumor antigens and the immunosuppressive tumor microenvironment (TME) limits ACT applicability. We combined the analysis of published datasets with a multidimensional investigation of CRC and PDAC samples collected and analyzed at Ospedale San Raffaele to select a set of tumor-associated antigens (TAAs) to be targeted by ACT. By clonal tracking of TCR repertoire of T cells stimulated with autologous APCs loaded with the selected antigens, we isolated 6 TCRs specific for different TAAs, including HER2 and mesothelin. TCR-transgenic T cells tested against PDAC cell lines and CRC patient-derived organoids showed anti-tumor efficacy toward HLA-matched cells while sparing HLA-unmatched controls. CRISPR/Cas9 is often selected as a tool to genetically manipulate T cells to redirect their specificity and endow them with the ability to counteract the immunosuppressive TME. However, CRISPR/Cas9 activity can produce important off-target events at the genomic level, affecting the safety profile of therapeutic cellular products. To overcome these limitations, we optimized the genome editing approach of T cells by relying on a cytosine base editor, BE4max. We generated $HER2_{369\text{-}377}$-specific T cells deprived of the endogenous TCR and TIGIT, a key immunosuppressive molecule, exploiting CRISPR/Cas9 and BE4max. Both editing procedures proved highly efficient in simultaneously disrupting *TRAC*, *TRBC1*, *TRBC2* and *TIGIT* (mean 96.6% and 96.3% of TRAC disruption, 81% and 73.6% of TRBC1 disruption, 75% and 67.3% of TRBC2 disruption and 89.3% and 90.7% of TIGIT disruption with CRISPR/Cas9 and BE4max, respectively) without impairing T cells' memory phenotype differentiation nor their expansion abilities. Remarkably, we only detected translocations between our four target loci in CRISPR/Cas9 treated cells (up to 0.44%). Moreover, by performing ultra-deep whole exome sequencing of base edited cells, we observed that BE4max efficiently mediated gene disruption without introducing biologically relevant off-target mutations. In functional assays, $TIGIT_{KO}HER2_{369\text{-}377}$-redirected T cells engineered with BE4max showed superior abilities, compared to TIGIT competent T cells, in eliminating PDAC cell lines (PANC1, T3M4 and BxPC3) and CRC patient-derived organoids. Our findings suggest that base editors can generate cellular products with a better safety profile and promising therapeutic efficacy in gastrointestinal tumors.

224 CD19/BCMA Dual-Targeting CAR-T Cells Generated by Co-Transduction for the Treatment of Non-Hodgkin Lymphoma

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Introduction: A common mechanism of resistance to CD19 CAR-T cell therapy is the loss or downregulation of antigen expression. Simultaneous targeting of more than one antigen has been proposed as a strategy to overcome this challenge. However, lack of preclinical studies comparing side-by-side the approaches together with missing predictive assays challenge the development of optimized clinical candidates. Here, we propose the dual-targeting of CD19 and BCMA to treat B-cell non-Hodgkin lymphoma patients, as most B-NHL subtypes co-express both antigens. Our in-house developed CARs targeting CD19 (ARI0001) and BCMA (ARI0002) have proven safe and efficacious in the treatment of patients with B-cell malignancies and multiple myeloma, respectively. We hypothesized that the co-transduction of two lentiviral vectors (ARI0001 and ARI0002) is a feasible strategy to obtain CD19/BCMA dual-targeting CAR-T cells with potential for clinical translation. **Methods**: We developed the following dual-targeting approaches: 1) Co-transduction of both lentiviral vectors (named ARI0003), 2) Pooled single CAR-T cells, 3) Bicistronic CAR-T cells encoding both CARs on the same lentiviral vector separated by a T2A peptide and 4) Tandem and Loop-CARs composed of two scFvs in one CAR construct. All CAR constructs contain the intracellular domains of 4-1BB and CD3. To evaluate these strategies in models that mimic a potential lack of response or relapse due to CD19 low densities, we developed two different preclinical models. First, we generated a CD19low cell line of Burkitt lymphoma and second, we generated Patient-Derived Lymphoma Spheroids (PDLS) from B-NHL patients that relapsed after treatment with CD19 CAR-T cells. Finally, we compared these strategies in different *in vivo* settings, including a model of high disease burden and a model of secondary treatment after treatment failure with ARI0001. **Results**: First, we observed that bicistronic and tandem/loop CARs required four times more integrated DNA lentiviral vector copies than the rest of the strategies to obtain the same levels of CAR expression on the T cell membrane, raising potential safety concerns. In addition, tandem and loop CARs showed limited long-term *in vitro* killing and persistence, which might be due to complex conformations of its extracellular domain and steric impediment to antigen recognition, and therefore were not included in future characterizations. Long-term *in vitro* assays, coculture experiments with PDLS from patients who relapsed anti-CD19 therapy and conventional *in vivo* experiments revealed that the co-transduction and pool groups behaved very similarly, significantly outperforming ARI0001 and the bicistronic vector in terms of killing, proliferation and antitumor effects, especially in models of low CD19 densities. Importantly, ARI0003 preserved effective antitumor activity after treatment failure with ARI-0001 against a CD19low B-NHL xenograft and outperformed the pool in the control of disease recurrence. Based on these results, ARI0003 was finally selected as our clinical candidate. The production of five clinical-grade CAR-T cell products using CliniMACS Prodigy confirmed the feasibility and potential of translating this strategy to the clinic. **Conclusion**: In summary, we propose the co-transduction as an efficient strategy to obtain highly effective CD19/BCMA dual CAR-T cells. We are currently preparing the required documentation to get approval from the regulatory agencies to initiate clinical trials to treat NHL patients with this therapy.

225 High-Throughput Screen of Chimeric Antigen Receptor (CAR) T Cells with Native and Non-Native Costimulatory Domains

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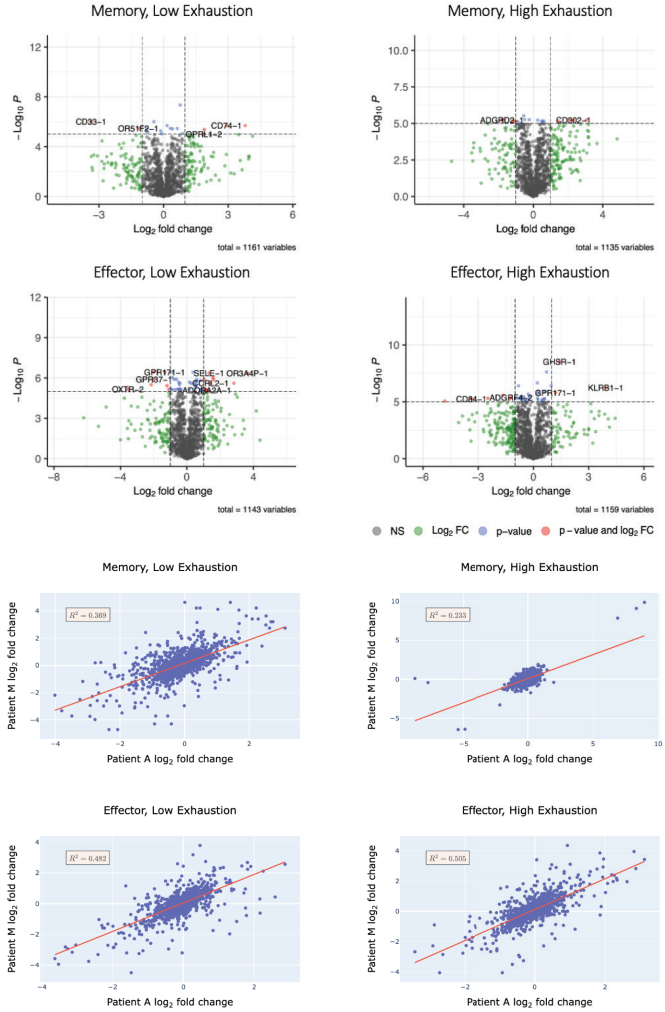
Chimeric antigen receptor (CAR) T-cells have shown great promise in clinical studies. Their therapeutic utility can be limited by lack of durability of the CAR T-cells and therapy related toxicities including cytokine release syndrome and neurotoxicity. Efficacy, persistence, and toxicity are known to correlate with the distribution of T cell subsets in the infusion product. T cell activation requires two signals, one that is antigen-dependent and another that in natural T cells is provided through the binding of a costimulatory receptor to its cognate ligand. In second generation CAR T cells, both signals are delivered by the CAR. Different costimulatory signals result in skewing of T-cell subsets. A limited variety of costimulatory domains have been used clinically and there has been relatively little research into the effect of non-native costimulatory domains. In this study we performed high throughput *in vitro* screening of > 1,200 intracellular domains from receptors found in the innate and adaptive immune systems for their potential utility to CAR T-cell therapy. To design the pool, we queried the Uniprot database for the primary sequences of the intracellular portions of transmembrane immune signaling molecules. Most of the elements in this pool are not naturally expressed in T cells and include receptors involved in activation, regulation, development and cytokine signaling of innate and adaptive immune cells. The motifs in our pool include those previously studied such as ITAMs and many not yet studied such as TIR and SPRY/B30.2. We inserted codon-optimized versions of the candidate costimulatory signaling sequences into a backbone plasmid containing a second generation anti-CD20 CAR with a truncated EGFR marker. We lentivirally transduced the pool of CAR into T cells from healthy human donors and co-cultured with CD20-expressing Raji cells. After 5 days the CAR T cells were FACS sorted by memory or effector T cell immunotype and high versus low expression of peripheral tolerance markers. We extracted DNA from the sorted CAR T cells and sequenced to identified costimulatory domains enriched in each group. The results were statistically analyzed to identify the enrichment or depletion of costimulatory domains which promote desirable T cell subtype distributions. We found that CAR T-cells with cytosolic portions of CD33 were depleted in the low exhaustion memory immunophenotype. CAR T with the cytosolic domain of KLRB1, an important receptor that inhibits cytotoxicity in NK cells, were enriched in CAR T with the high exhaustion effector phenotype. We performed our experiment with T cells obtained from two healthy human donors and found no significant differences in

the enrichment and depletion profiles. High throughput screening is a useful tool for CAR T-cell design. As a next step, we plan to use data generated by these experiments to train a machine learning model to predict the probability that a CAR T-cell with novel costimulatory receptors will adopt a desired immunophenotype.



226 Epitope Editing in Hematopoietic Cells Enables Universal Blood Cancer Immune Therapy

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Background: Targeted immunotherapies such as CAR-T cells are limited to several well-known lineage markers for which the on-target off-tumor toxicities are clinically tolerated. CAR-T cells have been developed individually for different hematologic malignancies based on the relevant lineage antigens (e.g. CD19 for B cell leukemias). By targeting a pan-hematologic antigen, a single "drug" could be used for all hematologic indications, thereby accelerating clinical research. CD45 is expressed on most hematologic malignancies and is, therefore, an attractive target for pan-hematologic cancer immunotherapy. However, targeting CD45 with CAR-T cells is limited by (i) on-target/off-tumor toxicities due to expression of CD45 on hematopoietic stem cells (HSC) and their progeny, and (ii) CAR-T cell fratricide. To protect HSCs and T cells from CART-45 mediated cytotoxicity we sought to modify the relevant CD45 epitope in T-cells and HSCs in such a way that it is unrecognized by the anti-CD45 CAR-T cells while preserving CD45's phosphatase activity, which is crucial for HSC and T-cell function. **Results:** We mapped the amino acid residues on the extracellular domain of human CD45 that are required for binding by anti-CD45 CAR-T cells (CART-45) by alanine mutagenesis and devised a CRISPR base editing approach to install a single mutation at the identified epitope. This allowed us to generate a CD45 molecule that is "invisible" to CART-45 cells. T-cells transduced with CART-45 show substantially impaired ex vivo expansion compared to CD19-directed CAR-T cells due to fratricide, as expected. However, CART-45 cells in which the targeted epitope was altered through adenine base editing (CART-45^{edited}) were resistant to fratricide, enabling their expansion comparable to that of CD45^{KO} or CAR19 control T cells. In contrast with CD45^{KO} CAR-T cells, CD45^{edited} cells retain CD45 phosphatase expression which we hypothesized to be crucial for CAR-T cell function in xenograft tumor models. In a B-ALL tumor xenograft model, CD45^{edited} CART-19 function was equivalent to CD45^{WT} CART-19, and superior to CD45^{KO} CART-19, indicating that CD45 is needed for the proper function of CAR-T cells. We then tested CART-45 in a patient-derived xenograft (PDX) model of human AML and found that CART-45 eliminated tumor cells with no detectable blasts in peripheral blood or bone marrow 4 weeks post CART-45 injection. To protect the hematopoietic system from CD45-directed on-target/off-tumor toxicity, we base-edited the targeted CD45 epitope in human HSCs, achieving the desired base conversion in 85% of human HSCs. CD45^{WT} and CD45^{edited} HSCs were engrafted into immunodeficient mice and treated with CART-45. Crucially, CD45^{edited} HSCs and their progeny persisted after CART-45 injection, whereas CD45^{WT} HSCs were readily eliminated, suggesting that CD45^{edited} hematopoiesis is protected from CART-45. We further show that CD45^{edited} HSCs transplanted into immunodeficient mice led to equivalent hematopoietic engraftment and differentiation when compared with CD45^{WT}cells, whereas CD45^{KO} hematopoiesis was lost over time. **Conclusions:** Here we developed a novel platform that combines pan-hematologic anti-CD45 CAR-T cells with an engineered hematopoietic stem cell transplant endowed with selective resistance to CD45-specific immunotherapy while maintaining CD45 expression and function. This platform creates a cancer-specific antigen in all residual host hematopoietic cells, enabling its use in most hematopoietic malignancies and perhaps other diseases requiring profound hematopoietic ablation.

227 Co-Expression of the IL15-IL15α Complex with a 41BB-Based Chimeric Antigen Receptor Promotes Superior Antitumor Activity in NKT Cells

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Despite successes in hematologic malignancies, conventional T cells expressing chimeric antigen receptors (CARs) are largely ineffective against solid tumors. Invariant natural killer T cells (NKTs) are a subset of innate lymphocytes that have inherent antitumor properties and traffic to tumor sites. We have shown that CAR-NKTs targeting disialoganglioside GD2 expressed by neuroblastoma (NB) tumor cells mediate potent antitumor activity in a xenogeneic model of NB. This effect was further enhanced by co-expression of the NKT homeostatic cytokine, IL15. Previous research has shown that *trans*-presentation of IL15 by IL15 receptor α (15Rα) is the main physiological mechanism of action for this cytokine. Therefore, we hypothesized that co-expressing IL15 and 15Rα (15.15Rα) in GD2.CAR NKTs will enhance their antitumor activity. We generated GD2.CAR constructs encoding the CD28 or 41BB co-stimulatory domain and co-expressed 15.15Rα (28z.15.15Rα and BBz.15.15Rα), using constructs without cytokine co-expression or co-expressing IL15 only as controls. We found that all constructs mediated similar levels of GD2-specific *in vitro* cytotoxicity against NB cell lines. However, BBz.15.15Rα NKTs had the greatest fold expansion after serial tumor challenge and crucially demonstrated superior control of tumor growth in a mouse model of NB, significantly increasing animal survival beyond all other groups ($p<0.01$). Gene expression analysis after NB tumor cell challenge revealed that BBz.15.15Rα NKTs have a similar gene expression profile to naïve or central memory T cells while 28z.15.15Rα NKTs instead resemble exhausted T cells ($p<0.05$). Therefore, the BBz.15.15Rα construct has emerged as the leading candidate for the next-generation CAR-NKT cell therapy for NB.

Gene Targeting and Gene Correction: New Technologies

228 Shuttle Peptide Delivers Base Editor RNPs to Rhesus Monkey Airway Epithelial Cells *In Vivo*

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Gene editing strategies for cystic fibrosis are challenged by the complex barrier properties of airway epithelia. We previously reported that the amphiphilic S10 shuttle peptide non-covalently combined with CRISPR-associated (Cas) ribonucleoprotein (RNP) enabled editing of human and mouse airway epithelia. Here, to improve base editor RNP delivery, we optimized S10 to derive the S315 shuttle peptide with improved Cas9 RNP delivery properties. Following intratracheal aerosolization of a Cy5-labeled peptide cargo with a nuclear localization signal in young rhesus monkeys, we confirmed delivery throughout the respiratory tract. A nuclear Cy5 signal was observed in luminal ciliated cells (acetylated tubulin$^+$), secretory cells (SCGB1A1$^+$), sporadically in basal cells (CK5$^+$), and in alveolar type II (SP-C$^+$) cells in the parenchymal regions. The delivery efficiency ranged between 0.5% - 20.8% and 1% - 17.8% in the large and small airways, respectively. Subsequently, we targeted the rhesus *CCR5* locus with co-administration of ABE8e-Cas9 RNP and S315 peptide by intratracheal aerosol. We achieved editing efficiencies up to 5.3% in rhesus airway epithelia (range 0.2% - 5.3%). The magnitude of Cy5 delivery and *CCR5* editing efficiency correlated with the anatomical distribution of deposited aerosolized materials as assessed by analysis of chest CT scans taken pre- and post-delivery. To investigate the long-term persistence of gene edited airway epithelial cells *in vivo*, we used the Ai9 ROSA26 tdTomato reporter mouse model. Following delivery of MAD7 nuclease RNP with the S10 shuttle, we documented persistence of edited tdTomato positive epithelial cells for up to 12 months (duration of the experiment). Finally, we examined the utility of the peptide-mediated base editor delivery to correct the CFTR function in human airway epithelia. We delivered ABE8e-Cas9 RNP with the S315 peptide to cultured human airway epithelial cells with the R553X mutation. We achieved 4.9% A•T to G•C editing at the target locus and restored CFTR-mediated anion secretion as measured in Ussing chambers. In summary, a single shuttle-peptide mediated delivery of ABE8e-Cas9 RNPs to the airways of the young rhesus monkeys, and *in vitro* delivery of the editor to human airway epithelia with the R553X mutation yielded similar editing efficiencies. In human CF epithelia adenine base editing restored CFTR anion channel function. These results demonstrate the therapeutic potential of base editor delivery with S315 to functionally correct the *CFTR* R553X mutation in human respiratory epithelia.

229 A Novel Type V CRISPR System with Potent Editing Activity in Mice and Non-Human Primates

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CRISPR-Cas based genome editing systems are being used extensively to make specific changes to the genomes of mammalian cells with the eventual goal of treating a wide range of genetically driven diseases. The majority of gene editing tools are based on the spCas9 or saCas9 nucleases which are members of the type II family of CRISPR systems. The type V family of CRISPR systems are structurally and functionally distinct enzymes that utilize a short guide RNA and generate staggered cuts. Despite the observation that type V CRISPR systems can exhibit higher specificity than type II systems, there is limited experience applying them for in vivo gene editing. The relatively low potency of native Cas12a/cpf1 has limited it's utility, and engineering approaches have yet to adequately address this. We discovered a new type V system designated MG29-1 from a metagenomics sample taken from a deep sea vent that exhibits minimal sequence identity to Cas12a/cpf1. MG29-1 is a 1280 amino acid RNA programmable nuclease that recognizes the PAM KTTN. Optimization of the guide RNA structure and chemical modifications, together with sequence optimization of the mRNA for MG29-1, enabled saturating editing in the liver of mice after systemic administration of mRNA and guide RNA encapsulated in a LNP, without the need for engineering of the nuclease. Guides for an exemplary target gene were identified that resulted in potent protein reduction in mice following single administration of LNP demonstrating the potential of MG29-1 for therapeutic gene knockdown. Two potent human/NHP guide RNA spacers were each co-formulated with the MG29-1 mRNA in a hepatocyte targeted LNP and infused into cynomolgus macaques. On-target editing as high as 55% in the whole liver (estimated to be 75% of hepatocytes) was achieved without notable safety concerns. While a transient elevation of ALT and AST was observed that returned to baseline by 4 days post dose, there was no significant increase in inflammatory cytokines despite the fact that no anti-inflammatory medications were used. Preliminary analysis of the off-target profile of MG29-1 using several guides targeting different genes supports the high fidelity of this nuclease. These results support further development of MG29-1 for therapeutic gene knockdown.

230 An *In Vivo* CAST-Seq Workflow Identifies and Quantifies Off-Target Activity as Well as Chromosomal Translocations in Organs Edited *In Vivo* with CRISPR-Cas Nucleases or Nickases

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Genome editing-associated genotoxicity, such as off-target (OT) activity and chromosomal translocations, must be carefully assessed before clinical application of gene-edited products. Three methods have been employed to evaluate OT activity *in vivo*: VIVO based on *in vitro* identification of candidate OTs that must be validated *in vivo*, DISCOVER-Seq relying on ChIP to nominate OTs based on the recruitment of MRE11 to double-strand breaks, and GUIDE-tag requiring the co-delivery of a DNA donor to 'tag' DSBs for subsequent capture. We recently described CAST-Seq, a method to nominate OT activity by detecting CRISPR-Cas induced chromosomal aberrations. Here, we show that *in vivo* CAST-Seq can identify OT sites and chromosomal translocations in *in vivo* edited mouse livers with unprecedented sensitivity. CAST-Seq was performed on genomic DNA isolated from livers upon adenoviral or AAV-based delivery of different CRISPR-Cas nucleases or nickases, respectively. For instance, OT analyses of *Pcsk9*-edited livers confirmed that CAST-Seq can identify more OT effects than the other three methods both quantitatively and qualitatively. While VIVO discovered 19 OTs, DISCOVER-Seq 26, and GUIDE-tag 40, CAST-Seq nominated 87 confirmed OTs. Subsequent analysis by rhAmp-Seq verified mutagenesis at these OTs, allowed us to establish the frequencies of chromosomal translocations, and demonstrated that each OT translocated with at least one other OT site. In addition, for a highly specific *Pcsk9*-targeting CRISPR-Cas9 nuclease previously reported to have no off-target activity, 4 OT-mediated translocations could be identified, despite a very low percentage of indels at these OTs (0.1-0.23%). Importantly, on-target aberrations, such as large deletions or integrations of vector sequences, could be readily detected using *in vivo* CAST-Seq. In conclusion, CAST-Seq followed by rhAmp-Seq provides a workflow to identify and quantify genome editing-associated OT activity as well as gross chromosomal rearrangements *in vivo*, with a process that is applicable to any tissue at any time point after editing, including human biopsies during therapeutic *in vivo* genome editing.

231 Writing a Chimeric Antigen Receptor (CAR) into T Cell Genomes Using RNA-Based Gene Writing Systems

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Chimeric Antigen Receptor (CAR)-T cell therapy is a revolutionary approach to cancer treatment where patient's autologous T cells are modified to express a CAR specific for a tumor antigen and kill the malignant cells. As of today, all approved CAR-T cell treatments use vectors of retroviral origin to deliver the CAR transgene. While these therapies have proven to be effective, the use of retroviruses poses several challenges. The complex manufacturing process reduces scalability with consequent limited access to this therapy. There are also safety concerns given the potential for formation of replication competent viruses, as well as the integration profile that is biased towards gene bodies. Our RNA Gene Writer technology leverages target-primed reverse transcription (TPRT) biochemistry evolved from non-LTR retrotransposon mobile genetic elements to modify the genetic information in cells without the introduction of DNA breaks. Using a large throughput screening approach, we developed multiple synthetic Gene Writer enzymes that can catalyze a variety of editing reactions, such as the introduction of gene-length DNA sequences, substitutions, insertions, and deletions. These edits can be achieved with all-RNA delivery in primary cells and *in vivo*, eliminating the necessity for viral vectors and DNA template-based gene editing. When using this novel class of gene editing enzymes, our Gene Writer technology can modify up to 40% of primary human T cells to express a transgene. This process was well-tolerated with little impact on cell viability, proliferation, and activation of p53 or interferon responses and is compatible with lipid nanoparticles (LNP) delivery. RNA Gene Writer enzymes delivered via electroporation can re-engineer T cells to express a functional CAR and kill tumor cells *in vitro* and *in vivo*, comparable to a lentivirus application. In addition, the modularity of our RNA Gene Writing technology allows multiplex editing to co-introduce multiple genetic changes including generation of universal and more potent CAR-T cells through the knock-out of B2M and TRAC without generating double strand breaks. Here we show that we can achieve both edits simultaneously in 80% of T cells while concurrently integrating a CAR transgene via another RNA Gene Writer enzyme. Furthermore, we will show successful LNP-based delivery of RNA to T cells *in vivo*, 80% reporter expression in a humanized mouse model and 45% in non-human primates (NHP) that may facilitate generation of CAR-T cells *in vivo*. As of today, our RNA Gene Writing technology is uniquely positioned for a one-time delivery of all-RNA components *to* achieve wide editing capabilities, from gene knock-out to gene integration. This technology will enable a simpler manufacturing process and a broader application for immunotherapies.

232 Cell Type-Programmable CRISPR-Cas9 Delivery for Human T Cell Engineering

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The lack of approaches for delivering genome editors to specific cell types limits the therapeutic application of CRISPR-Cas9. Enveloped delivery vehicles (EDVs), such as virus-like particles, enable targeted genome editing by leveraging the cell binding specificity of viral glycoproteins for the transient delivery of CRISPR-Cas9 tools. However, the repertoire of naturally occurring viral glycoproteins with cell-specific tropisms (ex. HIV-1 envelope glycoprotein to target CD4+ T cells) is limited. Inspired by chimeric antigen receptors and their ability to link programmable targeting to cellular function, we hypothesized that displaying antibody fragments on Cas9-guide RNA complex-packaging enveloped delivery vehicles (Cas9-EDVs) would allow for the delivery of genome editors to specific cells. Working with engineered 293T cells, we found that antibody-targeted Cas9-EDVs direct genome editor delivery to cognate ligand-expressing cells, leaving bystander cells largely unmodified. We next screened a panel of antibody-based molecules for genome editing primary human T cells. We identified Cas9-EDV targeting molecules for specifically editing T cell subpopulations (anti-CD4) and T cells broadly (anti-CD3). CD3-targeted Cas9-EDVs mediate genome editing levels comparable to Cas9-EDVs pseudotyped with the broadly-transducing VSV-G viral glycoprotein. Additionally, this antibody-directed targeting approach can be applied to lentiviruses for precisely delivering transgenes to T cells, both ex vivo and in vivo. Retro-orbital administration of CD3-targeted lentiviral EDVs leads to the generation of chimeric antigen receptor (CAR) T cells in humanized mice, demonstrating targeted delivery in vivo. Together, antibody-targeted EDVs are a programmable approach for the transient, cell-selective delivery of transgenes and CRISPR-Cas9 genome editor complexes.

233 Efficient and Safe RNA Editing in Non-Human Primates Using AAV Delivered LEAPER Agents

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Programmable A to G editing could theoretically treat nearly half of the genetic diseases caused by single-nucleotide polymorphisms (SNPs), including those leading to premature stop codons or splicing site mutation. As an important post-transcriptional modification, adenosine deaminases acting on RNA (ADAR) mediated RNA editing occurs widely in eukaryotic cells. In this process, the adenosine undergoes deamination to become inosine, which is recognized as guanosine in subsequent splicing or translation mechanisms, thus enabling the rewriting of the genetic message. Several ADAR-dependent RNA base editing tools have been developed. Among them, LEAPER and its upgraded version, LEAPER 2.0, enables precise, efficient, and

long-lasting editing of RNA transcripts with engineered circular arRNAs (circ-arRNAs) and endogenous ADAR. However, it is unclear whether such an approach is realistic for therapeutics given that the expression of endogenous ADAR varies with organs and species, and the choice of delivery routes has not been thoroughly validated. Here we report that LEAPER 2.0 enables efficient and safe RNA editing in non-human primates (NHPs) delivered by AAV. Specifically, we tested three doses, 3×10^{12}, 1×10^{13}, and 3×10^{13} vg/kg to deliver circ-arRNA into NHPs and the overall editing efficiency reached ~50% in liver within 1-3-month post infection. The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity levels, body weights and cytokine analysis in the experimental groups were all comparable to the control group, and the liver slices showed no liver injury throughout the experiment. To test whether circ-arRNA-mediated targeted RNA editing affects endogenous ADAR activity, we performed AEI (Alu editing index) assay in two 3×10^{13} vg/kg NHPs and the control group. The results showed no significant difference in AEI between them, indicating that the endogenous cellular ADAR editing was not affected. The specificity of endogenous ADAR-based RNA editing has been reported at the transcriptome-wide level in comparison with $ADAR2_{DD}$ (ADAR2 deaminase domain) overexpression-based RNA editing. We performed again such transcriptome-wide RNA sequencing analysis in NHP livers and detected only 18 potential off-target sites, and most of them either were located in the 3' UTR regions or caused synonymous mutations. The minimum free energy analysis suggested none of these off-target hits form a stable duplex with circ-arRNA. Therefore, it is unlikely that any of these sites were sequence-dependent off-targets. In summary, our results demonstrate that LEAPER 2.0 via AAV delivery enable efficient editing in non-human primates with potential long-term persistence, showing broad applicability for both therapeutics and biomedical research.

234 Co-LOCKR-Mediated, Highly Specific *In Vivo* Targeting without Off-Targets

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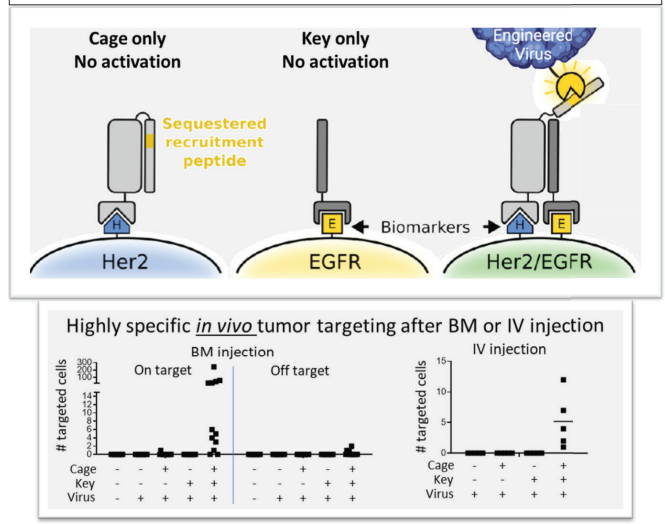
Gene therapy is currently performed *ex vivo*, which requires extensive medical infrastructure, limiting the availability. Moving to *in vivo* therapies would avoid infrastructure limitation and enhance feasibility, portability, and access. A key bottleneck for *in vivo* delivery is the target specificity to avoid unwanted off target effects. Agents recognizing only a single marker on target cells are often insufficient due to epitope sharing in between on- and off-target tissues. The Baker Lab previously designed a system of protein switches known as Co-LOCKR, which enable the specific recognition of two antigens simultaneously. Co-LOCKR targeted CAR T cells highly specifically killed tumor cells *in vitro* with virtually no off-target effects. Here, we designed and evaluated Co-LOCKR-targeted viral vectors for *ex vivo* as well as *in vivo* applicability and comprehensively tested the on-target specificity in a murine tumor model. We initially designed a modified lentiviral vector incorporating the BCL-2 peptide on the capsid to enable the recognition of the sequestered peptide Bim embedded in the Cage protein. We modified the VSVG fusion protein and knocked out its native ability to target low density lipoprotein ensuring the virus could only bind cells recognized by both components of the Co-LOCKR complex. Viral particles were quality controlled for size, concentration, and binding ability to Bim using in-house developed nanoparticle tracking analysis (NTA)-based read out. Specificity and functionality of viral particles was initially tested on cell lines ex vivo. Once this was confirmed, we moved to in vivo tumor targeting. Ex vivo, BCL-2 targeted vectors specifically transduced $EGFR^+HER2^+$expressing Raji cells in the presence of both the cage and key. No off-target was seen either in $EGFR^-Her2^-$ cells or in the absence of individual Co-LOCKR components. Next, we co-injected both cell types into femoral bones of irradiated NBSGW mice. Once the tumor was established, BCL-2 targeted vectors and Co-LOCKR proteins were injected into the tumor-infiltrated bone. Similar to the ex vivo experiments, we observe highly specific targeting of $EGFR^+HER2^+$ expressing Raji cells only in the presence of all Co-LOCKR components and virtually no off-target. Finally, we injected the virus and protein into the tail vein to evaluate the feasibility of our system for a systemic administration. Although the targeting efficiency decreased, highly specific on-target transduction in $EGFR^+HER2^+$ expressing Raji cells was seen. Here we show that Co-LOCKR proteins combined with engineered viral vectors can localize target cells ex vivo as well as in vivo with a remarkably high on-target specificity. Co-LOCKR proteins and Bcl2-targeted viral vectors are stable, resist serum inactivation, and can travel the full length of a murine circulatory system, enter the bone marrow, and successfully transduce cells even in hardly accessible tissues. Most importantly, Cage and Key targeting molecules can easily be exchanged for the targeting of hematopoietic stem cells or T cells. The ability to target bone marrow-resident cells highly specifically via an intravenous administration of this novel delivery system provides a new strategy to perform HSC gene therapy in vivo without the need of mobilization.

Figure: Diagram of Co-LOCKR system functionality. Co-LOCKR components and viruses injected either directly into the bone marrow compartment or through tail vein of mice specifically target cells of interest.



Pharmacology/Toxicology Studies: Bio Distribution

235 PK/PD Modeling to Inform Clinical Development of an Adeno-Associated Virus Gene Transfer Therapy for Duchenne Muscular Dystrophy

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Delandistrogene moxeparvovec (previously known as SRP-9001) is an investigational recombinant adeno-associated viral (rAAV) vector-based gene transfer therapy designed to compensate for missing dystrophin in Duchenne muscular dystrophy (DMD) by delivering the SRP-9001 dystrophin transgene, which encodes a shortened, engineered dystrophin protein that retains key functional domains of the wild-type protein. We evaluated vector biodistribution, expression, and clinical dose selection of delandistrogene moxeparvovec using a novel application of a pharmacokinetic (PK) and pharmacodynamic (PD) modeling approach applied to data collected from an animal model of DMD (DMD^{MDX} mice). We analyzed PK/PD relationships between dose, tissue vector genome exposure, micro-dystrophin protein expression (percentage dystrophin-positive fibers [PDPF] and western blot), and functional improvement (relative specific force from tibialis anterior and diaphragm). Linear kinetics with a dose-proportional increase in tissue drug exposure were demonstrated across the nearly 10-fold dose range (4.41x1013-4.01x1014 vg/kg), and in all tissues. The relationship between tissue vector exposure and PD endpoints (PDPF, motor function outcome) showed a saturable response across a wide range of vector exposures. The vector exposure at 1.33x1014 vg/kg (the clinical dose) approached the maximum treatment response. Relative specific force and PDPF were significantly correlated (P=4.43x10-6). However, the relationship appeared to be nonlinear, with increased PDPF expression approaching the maximal functional improvement. Relative specific force and western blot were not significantly correlated. For the first time, biodistribution, biomarker, and functional efficacy data were used to quantify and demonstrate PK/PD relationships for an adeno-associated virus (AAV)-based gene transfer therapy in a DMD animal model. The results continue to support the expected therapeutic benefit and clinical dose of delandistrogene moxeparvovec, an AAV-based gene transfer therapy. This study was funded by Sarepta Therapeutics, Inc.

236 Vector Shedding in Patients with DMD Treated with Delandistrogene Moxeparvovec and Seroconversion from Shed Vector in Naïve Mice

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Delandistrogene moxeparvovec (previously known as SRP-9001) is an investigational gene transfer therapy designed to compensate for missing dystrophin in Duchenne muscular dystrophy (DMD) by delivering the SRP-9001 dystrophin transgene, which encodes a shortened, engineered dystrophin protein that retains key functional domains of the wild-type protein, packaged in an adeno-associated virus (AAV) vector. Due to the biological properties of viruses, vector shedding is expected following the administration of a virus-based gene therapy product. We evaluated the extent of vector shedding and clearance following a single administration of delandistrogene moxeparvovec (1.33×10^{14} vg/kg body weight) in ENDEAVOR (NCT04626674; Study 103; N=20), a Phase 1 study in patients with DMD. Delandistrogene moxeparvovec vector exposure in saliva, urine, and feces was quantified by droplet digital polymerase chain reaction. In a nonclinical study, we tested naïve mice to determine the risk of AAVrh74 seroconversion following mucosal vector exposure, with doses based on exposure levels (vector genome copies) demonstrated in nonclinical and clinical studies. Mice were exposed to AAVrh74.CMV.eGFP via optic exposure and intramuscularly. Antibody levels were measured by AAVrh74 ELISA at baseline and 4 weeks post-delivery. Following treatment in ENDEAVOR, the percentage decrease in amount of vector shed was >99% in saliva (n=12), urine (n=18), and feces (n=11) by Week 4. In mice, topical optical delivery of the AAVrh74 vector at relevant concentrations did not produce seropositivity or detectable vector genomes throughout tissues at doses based on clinical vector shedding levels. Clinical findings show that peak vector shedding occurs in the first few days after delandistrogene moxeparvovec administration and exponentially declines to insignificant levels by Week 4. In mice, seroconversion was not observed by relevant exposure route at relevant concentrations. Results suggest that the risk of seroconversion following exposure to vector shed by individuals treated with AAVrh74-based gene therapy may be very low. These conclusions conform with data and literature for other AAV-based gene therapy products currently approved or in development. This research was funded by Sarepta Therapeutics.

237 Development of an Immunosuppressed Murine Model for AAV Based Gene Therapy Biodistribution Studies

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In the past 6 years, 3 adeno-associated virus (AAV) based gene therapy products were approved by the United States Food and Drug Administration (USFDA), and it is anticipated that up to 20 products will be approved per year by 2025. This necessitates generating models more comparative to first in human administration scenarios, for which most preclinical studies conventionally use non-human primates (NHPs) in safety assessment. Due to the global shortage of captive-bred NHPs, the USFDA has provided a rich climate for considerations to alternate models via guidance, required for the success of gene therapy product development. In this work we describe our unique first-tiered approach to applying standard immunosuppression (IS) protocols used in NHPs to rate AAV gene therapy safety in a murine surrogate. These models represent alternatives to NHP models for general preclinical safety studies to continue to inform about risks in the advancement of new treatments. Many benefits exist in the development of an alternate IS murine model that may outweigh those of using NHPs. The murine model is competitively cost effective, rodents are easy to handle, require smaller housing facilities, and are readily available in large numbers. Additionally, murine models allow flexible experimental design, such as group numbers and increased number of endpoints, that are advantageous to discovery and preclinical therapeutic development. Further, the IS murine model reflects a real world scenario where immunocompetent patients require immunosuppression in order to limit uncontrolled inflammatory and cytotoxic responses from the immune system. For example, gene therapy patients who are administered Zolgensma or Luxterna receive systemic corticosteroids (Prednisolone) prior to AAV gene therapy product administration.In this 12-week study, we investigated the AAV2-mCherry and AAV9-mCherry vector biodistribution in C57BL/6J immunosuppressed murine model following an intravenous vector administration at a dose of $1x10^{14}$ genome copies/kg on Day 15 while the animals received Azathioprine or Prednisolone from Day 1 through Day 84 to assess the safety profile and tissue-specific transduction efficiency. The level of immunosuppression in the murine model was evaluated by $CD3e^{-}/CD19^{+}$ B Cell count in the peripheral blood once every 4 weeks, starting on 1st day of immunosuppressant dosing. The B Cell count demonstrated the dynamic nature of adaptive immune system, where the B cell population fluctuated or came in waves even when the dose of the immunosuppressant was stable throughout the regimen. In comparison, biodistribution models in immuno-incompetent models like NSG or SCID mice would not capture this dynamic immune response. We also evaluated the tissue-specific transduction efficiency by qPCR in the brain, lumbar dorsal root ganglia, heart, kidney, liver, lung, cervical, thoracic and lumbar spinal cord, spleen, thymus and ovary/testes of the female and male immunosuppressed animals. Vector transduction was the highest in the liver, followed by kidney and heart tissue in both the AAV2-mCherry and AAV9-mCherry administered immunosuppressed groups. Moderate level of transduction was seen in the brain and ovary/testes of AAV9-mCherry dosed animals, whereas low level of transduction was observed in the lumbar dorsal root ganglion of AAV2-mCherry and AAV9-mCherry animals.Taken together, our data demonstrates the feasibility of using IS murine models in AAV based toxicology and biodistribution studies. The development and communication of the IS murine model is a promising path forward in the safety assessment of gene therapies.

238 Assessment via Positron Emission Tomography of Adenovirus Capsid Biodistribution Following Intramuscular versus Intravenous Administration to Nonhuman Primates

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Adenovirus (Ad)-based vaccines are clinically approved for Ebola and COVID-19, and in various stages of development for a variety of other disorders. Despite the clinical use of these vaccines, there is little quantitative information available regarding adenovirus organ biodistribution following administration, especially in the event that an intended intramuscular Ad vaccine is inadvertently administered intravascularly. Using Ad serotype 5 (Ad5) covalently linked to the positron emitter iodine-124 (I-124, 4.2 day half-life) to enable positron emission tomography (PET), the goal of this study was to assess Ad biodistribution *in vivo*. As a first step, we assessed the distribution of Ad5 following intramuscular (IM) administration compared to the scenario of inadvertent intravenous (IV) administration. African Green nonhuman primates (NHP) were administered 0.37 - 2.96 MBq (10 - 80 μCi) of ^{124}I-Ad5, either intravenously in the right saphenous vein or intramuscularly in the deltoid muscle. The deltoid was chosen to mimic the most common site of human vaccination. Animals were imaged under anesthesia in an extended field of view (FOV = 4 ring, 22 cm) Siemens Biograph PET/CT immediately after injection and at 24, 48, and 72 h post-injection (p.i.). Coregistered CT's were acquired for attenuation correction and anatomical information. The reconstructed PET resolution was 2.0 x 2.0 x 5.0 mm^3. Regions of interest (ROI) were drawn over the heart, liver, spleen, brain, thyroid, PET-visible lymph nodes, deltoid muscle, kidneys, and whole body. Time-activity curves were fitted to a systemic biodistribution model to calculate the fractional biodistribution to ROI's and biological clearance times. The largest proportion of activity after IM administration was initially at the injection site as expected. In addition, two axillary lymph nodes nearest to the injection were clearly visible and exhibited high activity at all timepoints. Besides the injection site and associated lymph nodes, peak concentration values were in the gall bladder at 0.09% injected dose per gram (ID/g), and kidney at 0.04% ID/g, suggesting a renal route of Ad clearance. Tissue biodistribution patterns were markedly different with IV administration. Early systemic distribution was generally higher for all organs in the IV case by a factor of 2 or more at 0 h p.i. with the exception of kidney which was similar (1.4% ID/g

IM, 1.6% ID/g IV). Peak concentration values for IV administration were in the liver and spleen (0.24% ID/g and 0.17% ID/g respectively). Liver activity was exceptionally disparate, with peak activity occurring within 1 h p.i., and accounted for 37.7% of the total ID for IV administration, but only 1.8% ID following IM administration. Thyroid accumulation also increased, as expected, across all days for both IM and IV administration, peaking at similar values (5.3% ID/g *vs* 3.8% ID/g respectively at 72 h p.i). This is hypothesized to be the result of extraction from the bloodstream of free I-124 resulting from capsid processing. Although overall biodistribution patterns were very dissimilar, whole body clearance halftime was 18.2 h for IM and 17.0 h for IV. In summary, the biodistribution of Ad5 in NHP when administered via IM *vs* IV injection is significantly different across almost all organs as detected *in vivo* using radioiodinated capsids and PET. This methodology facilitates quantification of vector organ uptake and can be used to assess risk associated with different vaccine administration routes.

239 Neural Stem Cell Mediated Oncolytic Virotherapy for Ovarian Cancer

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Introduction. Oncolytic virotherapy is a promising cancer treatment that uses replication-competent viruses to induce cancer cell death. While clinical trials are underway for a variety of solid tumors; success has been hampered by rapid immune-mediated clearance/neutralization of the viral vectors, and poor viral distribution to tumor satellites dispersed throughout normal tissue. Neural stem cells (NSCs) are ideal cell carriers that could overcome viral delivery hurdles due to their intrinsic tumor-tropism and penetration capabilities. Our lab has established a well-characterized, non-immunogenic human NSC line that can selectively distribute to many different solid tumors. Most recently, we observed impressive selectivity and penetration of peritoneal ovarian cancer metastases after intraperitoneal NSC administration. We have engineered our NSCs to produce a conditionally replication-competent adenovirus, CRAd-Survivin-pk7 (CRAd-S-pk7 NSCs). This virus has two notable genetic modifications: (1) a polylysine fiber addition that enables high affinity binding to cell-surface proteoglycans, thus promoting viral entry into the target cell; and (2) a E1A transcriptional modification which prevents viral replication in the absence of the *survivin* promoter. 80% of ovarian tumors have elevated levels of survivin, which then drives viral replication. Clinical grade equivalent research banks of the CRAd-S-pk7 NSCs cells have demonstrated efficacy in orthotopic glioma models (IND 19532), and safety in a recent first-in-human trial (NCT 03072134) but have not yet been developed for treating metastatic ovarian cancer. We hypothesize that NSCs can selectively distribute this virus to ovarian metastases, provide protection from immune-mediated clearance, and achieve significant improvements in long-term survival. Our long-term goal is to demonstrate efficacy and safety of CRAd-S-pk7 NSCs for targeted selective tumor killing in patients suffering from stage III ovarian cancer. ***Methods***. Human Xenograft and immunocompetent syngeneic models of peritoneal ovarian cancer were treated with escalating doses of CRAd-S-pk7 NSCs. Treatment efficacy was determined by week 8 tumor burden and long-term survival. Pilot toxicology studies were conducted using the highest and lowest effective dose. Immunophenotyping was performed on treated tumors to assess possible mechanism-of-action. ***Results***. We demonstrate that CRAd-S-pk7 NSCs can improve long-term survival in mouse models of ovarian cancer. We identify the LOAEL dose, demonstrated negligible toxicity at this dose, and confirm CRAd-S-pk7 NSC treatment causes t-cell infiltration into the tumor micro-environment (improves CD8+/FoxP3 ratio). The LOAEL dose was converted to human equivalent levels, and Pre-IND package submitted.

240 A Novel Technique to Detect Peripheral Blood CAR+ T Cells Using RNAscope *In Situ* Hybridization (ISH) in Non-Human Primates and Mice

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In clinical studies, CAR T cells are routinely detected in blood and/or tissues either by flow cytometry (using an anti-idiotype antibody) or by ddPCR. When preclinical studies are conducted in animal models, flow cytometry can be challenging due to limiting blood sample volumes or ability to obtain species specific reagents. We developed a novel method using HistoGel™ to facilitate the detection of either CD20-directed or CD19-directed CAR T cells in peripheral blood mononuclear cells (PBMCs) by *in situ* hybridization (ISH). HistoGel is an aqueous specimen-processing gel that suspends cytologic, i.e., cell culture or cytospin samples from body fluids, in a solidified medium amenable for histologic processing. HistoGel has the advantage that it also can be repeatedly sampled. Probes to detect woodchuck hepatitis post-transcriptional regulatory element (WPRE) mRNA were used to detect transcription of the WPRE element that is part of the polycistronic transcript that was used to encode each CAR. WPRE serves as a surrogate marker for engineered T cells expressing the CARs. We used CD8-targeted fusosomes to generate in vivo CAR T cells. These fusosomes are pseudotyped integrating viral vectors designed to specifically target CD8+ T cells and deliver a chimeric antigen receptor (CAR) transgene. Samples for evaluation were obtained from each of three studies. Studies 1 and 2: Nemestrina macques were intravenously administered a single dose of a CD8-targeted fusosome, carrying a CD20 CAR transgene and euthanized 8 days (Biodistribution Study) or 35 days (Safety and Pharmacology Study) after infusion. Following infusion, CD8+ enriched PBMCs were isolated on Days 2, 4 and 7, fixed in 10% formalin, centrifuged, and the resulting cell pellet was resuspended in HistoGel. The PBMC- HistoGel pellets were processed for paraffin embedding, sectioned, and stained by ISH. Study 3: NSG mice were engrafted with human PBMCs and 24-hrs later were administered a CD8-targeted fusosome carrying a CD19 CAR transgene as part of a pharmacology study. On Day 4, human PBMCs were isolated from 4 mice, pooled, and processed using the same HistoGelmethod. Bone marrow aspirates from a different set of NSG mice from the same study were harvested and processed in

HistoGel. ISH assay positive controls consisted of PBMC cell pellets from human and Nemestrina, that were transduced in vitro with the CD8-targeted CD19 CAR and CD20 CAR fusosomes, respectively. Expression of WPRE was observed in Nemestrina PBMCs as early as Study Day 2 as fine punctate to granular staining and also present in PBMCs on days 4 and 7. Similar to Nemestrina, WPRE expression was detected in human PBMCs isolated from NSG mouse samples on Day 7 and in bone marrow aspirates on Day 30. These data demonstrate for the first time the feasibility of using a HistoGelmethod for ISH on circulating and tissue derived PBMC and bone marrow aspirates to specifically detect CAR T cells. Although specificity or sensitivity of the HistoGelmethod was not compared to flow cytometry, the novel technique may have utility beyond preclinical research for application in the clinical laboratory setting where limiting cell samples or archival cell or tissue samples are available.

241 Nonclinical Pharmacology, Biodistribution, and Safety Studies Supporting the Clinical Development of DB-OTO (AAV1-Myo15-hOTOFv5) for Hearing Loss Due to Genetic Otoferlin Protein Deficiency

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Background Otoferlin is a calcium sensor protein expressed in the inner hair cells and is important for proper synaptic transmission between inner hair cells and the afferent fibers of the spiral ganglion. Biallelic loss of function mutations in the OTOF gene lead to congenital severe-to-profound sensorineural hearing loss in both humans and in mice. DB-OTO is an adeno-associated virus (AAV)-based dual-vector gene therapy designed to provide durable hearing to individuals with profound congenital hearing loss caused by mutations of the otoferlin gene. **Methods** Good Laboratory Practice (GLP) studies were performed to support the conduct of human clinical trials of DB-OTO. OTOF-deficient mice and wild type non-human primates (NHPs) were used to characterize the pharmacology, toxicity and biodistribution of DB-OTO after inner ear delivery. In the experiments involving NHPs, we modeled the surgical approach and delivery of DB-OTO that is being used in the first-in-human clinical trial. **Results** DB-OTO can instate hearing function in OTOF$^{Q828X/Q828X}$ mutant mice as measured by ABR and it can be successfully delivered to the NHP ear via RW injection with lateral semi-circular canal fenestration. Using qRT-PCR, we found that hOTOF mRNA transcript levels peaked 4 weeks after DB-OTO injection in mice and 6 weeks after DB-OTO injection in NHPs, plateauing thereafter. A similar time course was observed for ABR improvements post DB-OTO injection in the congenitally deaf OTOF$^{Q828X/Q828X}$ mutant mice. We followed OTOF$^{Q828X/Q828X}$ mice for 8 months post-administration of DB-OTO and observed stability of the instated ABR throughout that period at clinically relevant doses. In GLP studies, there were no adverse DB-OTO-related findings in otic or non-otic tissues across any evaluation in OTOF$^{Q828X/Q828X}$ mice (5-7-week-old and post-natal day 14-16) or healthy NHPs. We assessed the distribution of vector genomes outside of the ear following DB-OTO injection in NHPs. Vector shedding and levels of systemic escape into tissues in NHPs were limited. The proposed volumetric dose scaling was directly proportional to the total cochlea perilymph volumes of mouse, NHP, and humans. Comparable vector genome DNA and hOTOF mRNA levels in mouse and monkey GLP studies confirmed the validity of this volumetric scaling approach for dose adjustments of DB-OTO between species. The presence of pre-existing neutralizing antibodies were not associated with transgene expression in the ear or safety post DB-OTO injection, suggesting that there may be limited impact of systemic pre-existing neutralizing antibodies on local administration of AAV gene therapies to the inner ear. **Conclusions** Together, these data supported the initiation of a Phase 1/2 clinical trial of DB-OTO in pediatric patients with congenital hearing loss due to an otoferlin deficiency.

Emerging Preclinical Gene and Cell Therapy Approaches for Neurological Disorders

242 Transplantation of Wild-Type Hematopoietic Stem and Progenitor Cells Rescue Alzheimer's Disease in a Mouse Model and Highlights the Central Role of Microglia in Disease Pathogenesis

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Alzheimer's disease (AD) is the most prevalent cause of dementia, but still no effective treatment exists. The major hallmark of AD is significant neuronal degradation, inflammation, progressive memory loss, behavioral decline and an accumulation of extracellular β-amyloid (Aβ) plaque in the brain. Microglia have been implicated in AD pathogenesis, but their role is still matter of debate. Sustained microglia inflammation has been identified as a contributor to AD pathogenesis, as the release of inflammatory cytokines and chemokines increase Aβ production.Because hematopoietic stem and progenitor cells (HSPCs) differentiate into microglia-like cells, we believe that the WT HSPC transplantation will shed further information on the significance of microglia in familial AD pathogenesis and could potentially be used to as a therapy. We used 5xFAD mouse model of AD, which expresses mutant human APP and PSEN1 transgenes, and transplanted them with Sca1^{+} HSPCs isolated from WT GFP transgenic mice after lethal irradiation (5xFAD/WT HSPC). As a control we analyzed WT, lethally

irradiated WT transplanted with WT HSPCs, non-treated 5xFAD, lethally irradiated 5xFAD mice transplanted with 5xFAD HSPCs (5xFAD/5xFAD HSPC). At 4-month post-transplant, behavioral testing demonstrated that transplantation of WT HSPCs in 5xFAD mice led to the preservation of their memory, perception of risks, anxiety level, and locomotor activity as opposed to 5xFAD and 5xFAD/5xFAD HSPC mice. Amyloid beta (Aβ) deposition was also reduced in the hippocampus and cortex of 5xFAD/WT HSPC mice compared to controls. We then characterized the engraftment and differentiation of WT HSPCs in the brain of mice and found substantial engraftment of GFP$^+$ HSPC-derived cells. These GFP$^+$ cells were immunoreactive with Iba1, characterizing these cells as microglia-like cells. We also observed that transplanting WT HSPCs led to the significant reduction in microgliosis and inflammatory state of the brain by immunohistochemistry, qPCR and Elisa. Transcriptomic analysis also revealed significant decrease in gene expression related to "disease-associated microglia" in the cortex, and "neurodegeneration-associated endothelial cells" in the hippocampus of the WT HSPC-transplanted 5xFAD mice compared to diseased controls. Altogether, these data suggest that microglia-associated neuroinflammation in AD is an important feature of neurodegeneration and preventing microgliosis was sufficient to prevent the neurocognitive impairments in the 5xFAD mice. This work also shows that HSPC transplant represents a promising therapeutic avenue for familial AD.

243 Intracerebroventricular Delivery of SLC6A8 by Self Complimentary AAV9 Restores Brain Creatine and Reduces Hyperactivity in a Mouse Model of SLC6A8 Deficiency

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Solute Carrier Family 6 Member 8 (SLC6A8, creatine transporter) is an active Na+Cl- dependent transporter responsible for cellular uptake of creatine. SLC6A8 deficiency is an X-linked genetic disorder induced by loss of function mutations in SLC6A8 resulting in the inability of cellular creatine uptake and a severely depleted or absence of creatine in the central nervous system. Creatine plays a role in many cellular functions, most notably as a phosphate buffer for ATP and is critical in proper regulation of energetics and metabolism in the brain. Disease manifestation includes a wide range of symptoms including developmental delay, intellectual disability, motor function impairment, seizures, hyperactivity, and autism-like behavior. Murine models share some of these symptoms such as hyperactivity, memory deficits, and decreased creatine and creatinine in the central nervous system. Additionally, knockout (KO) mice have reduced body weight and muscle mass. The frequency of SLC6A8 deficiency is not well known but thought to be between 1-2% of all X-linked intellectual disability cases. Due to the minimal passive transport of creatine, supplementation has a minimal effect leaving clinicians with a lack of therapeutic options. Here we present both in vitro and in vivo data on the delivery of SLC6A8 as a potential gene therapy treatment for SLC6A8 deficiency. In vitro transfection of a codon optimized SLC6A8 plasmid carrying the scAAV9-SLC6A8 construct restored cellular creatine levels. Intracerebroventricular (ICV) injections allow for direct delivery of viral vector to the brain on postnatal day 0. An ICV dose of 2.5e10 vector genomes of scAAV9-SLC6A8 in KO mice resulted in significant increases in body mass (by 25 %) and a significant decrease in night-time activity (by 50%) relative to untreated KO mice 24 weeks post injection approaching the activity of wildtype (WT) mice. Both mid and caudal sections of the brain showed a 5-fold increase in creatine and creatinine concentration with the midsection of the brain approaching WT creatine levels (Figure1. A, C). This data shows great potential for AAV delivery of SLC6A8 as a treatment for SLC6A8 deficiency. Further studies are ongoing to determine optimal dose and time of administration. Our study forms a foundation for a potential gene therapy of this so far untreatable condition.

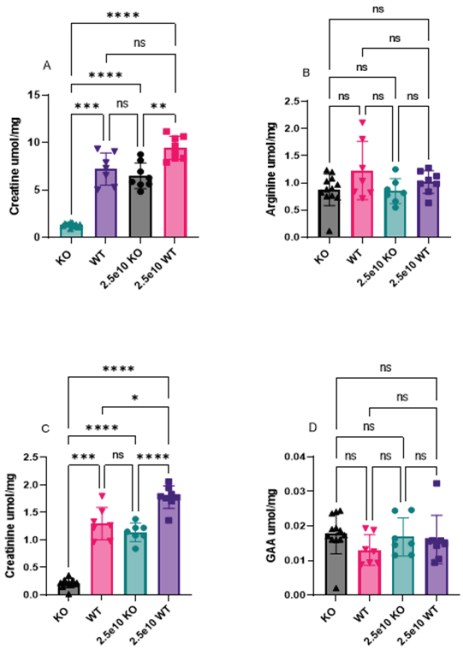

Figure 1. Postnatal day-0 intracerebroventricular delivery of 2.5e10 vg of *scAAV9SLC6A8* restores creatine concentration to KO mice in the midsection of the brain. Mice were euthanized 24 weeks after delivery and brain creatine, arginine, creatinine and Guanidinoacetate (GAA) were determined using liquid chromatography mass spectrometry and normalized to tissue mass. Both creatine and creatinine were significantly elevated in treated KO mice relative to KO counterparts (Dunnett T3 MC test * P<0.05, **P<0.01, *** P<0.001 ****P<0.0001). There were no statistical differences detected in either GAA or arginine concentration.

244 AAV9-Mediated Expression of Secreted Klotho Reduced Several Aging-Associated Phenotypes and Increased Longevity

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Advances in health care and quality of life in modern societies have led to an increase in the percentage of the population reaching advanced ages, with a projected 25 % of U.S. citizens being over the age of 65 by 2060. Aging is one of the main risk factors for a wide range of pathologies, including osteoporosis, sarcopenia, and cognitive degeneration. These conditions are accompanied by suffering, disability, and elevated economic and social costs, thus new therapies are needed to achieve healthy aging. The protein Klotho (KL) has been identified as a promising anti-aging molecule due to its pleiotropic actions, with pro-longevity effects on pathways such as insulin/insulin-like growth factor and Wnt signaling, and inflammatory and oxidative stress modulation. Here, we explored the anti-aging potential of the secreted isoform of this protein in the SAMP8 and C57BL mice, models for accelerated and non-pathological aging, respectively. Systemic and intracerebroventricular delivery of AAV9 efficiently increased concentration of s-KL protein in serum, improving the aging phenotype in different organs analyzed. KL treatment improved fitness in behavioral tests, associated with reduced muscular fibrosis and increased muscle regeneration. Cortical and trabecular microstructural parameters in the aged bones, measured by MicroCT analysis, were also improved in treated animals. Cognitive capacities of aged animals were improved, which was accompanied by increased markers of adult neurogenesis (Ki67 and DCX), and normalization of cellular markers in the hippocampus like Iba1$^+$ and GFAP$^+$ cells populations, supported by histological and transcriptional analysis. Remarkably, long-term AAV-mediated expression of s-KL lead to a 20% increase in total longevity of C57BL mice. These results show for the first time, the potential of exogenous increase in s-KL protein to treat several age-associated deficits, increasing both health and life span of wild type animals. Due to the safer pharmacological profile of s-KL compared to other KL isoforms, and being a naturally secreted protein, gene therapies increasing s-KL levels represent a promising approach to reduce the impact of the age-associated degeneration in multiple organs.

245 A System for Whole-Body Gene Delivery Rescues Wolfram Syndrome II

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Progerias result from mutations that cause accelerated onset of aging-like features in one or more of the body's organ systems. Wolfram Syndrome II is a rare human and mouse progeria characterized by sensorineural, metabolic and hormonal defects and caused by genetic disruption of the *Cisd2* gene. *Cisd2* is of considerable interest in the geroscience community, because its loss or reduction of expression leads to premature aging and has been associated with several prevalent human diseases, while its overexpression protects against age-related diseases and increases lifespan in mice. Gene therapies hold promise for treating progerias, but their application has been hampered by the fact that there are no known gene delivery vectors that can deliver DNA sufficiently broadly across the body. Here, we have engineered an adeno-associated virus (AAV) based system that enables efficient and tunable gene expression across multiple organs simultaneously. This system (called DAEUS) combines multiple engineered AAV serotypes and gene regulatory elements with model guided dosing. DAEUS can drive gene delivery to all tested organs, across more than 80% of the body mass overall. We used DAEUS to engineer a whole-body gene delivery to Wolfram Syndrome II. In Wolfram Syndrome II mice, DAEUS-*Cisd2* gene therapy restores near wild-type *Cisd2* expression across major tissues of the body and protects against development of disease in all cohorts tested. Specifically, mice treated as neonates or young adults, DAEUS-*Cisd2* therapy prevents the development of frailty, loss of activity, loss of vision and extends post-treatment lifespan by 75% to 140%. Surprisingly, in mice with advanced progeria, DAEUS-*Cisd2* gene therapy reverts disease pathology in multiple tissues, in addition to extending lifespan and protecting against further progression of the disease. These data indicate that DAEUS holds promise as a platform for treatment of multi-organ genetic diseases and that restoration of *Cisd2* expression may provide therapeutic benefit to Wolfram Syndrome II patients at any treatment age. This work was supported by the NIH DP1 AG063419 and Glenn Foundation

246 A Gene Therapy Approach to Treat PMLD1

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Pelizaeus-Merzbacher-like disease (PMLD1)/ hypomyelinating leukodystrophy type 2 (HLD2) is an autosomal recessive hypomyelinating leukodystrophy, which is characterized by early-onset nystagmus by 7 weeks of age, impaired motor development and ataxia, spasticity, dysarthria, dystonia, seizures, optic atrophy, and diffuse hypomyelination in MRI. PMLD1/ HLD2 is caused by loss-of-function mutations in the *GJC2/Cx47* gene encoding the gap junction (GJ) protein connexin47 (Cx47). Cx47 is expressed specifically in all oligodendrocytes throughout the CNS and is crucial for their gap junctional communication and homeostasis forming most GJs with

other oligodendrocytes and astrocytes. The aim of this study was to develop an optimal gene replacement therapy comparing lumbar intrathecal and intravenous delivery of two leading AAV serotypes, AAV9 and AAVrh10, that are currently used in most clinical trials for other neurological disorders. The 1.9kb myelin basic protein (Mbp) promoter was used to drive expression of the reporter gene EGFP or of the human *GJC2/Cx47* gene specifically in oligodendrocytes. To directly compare serotypes and routes of delivery, AAV9-*Mbp.GJC2*, AAVrh10-*Mbp.GJC2*, and the corresponding reporter gene vectors (titers of at least 1x10^13 vg/ml) were injected into P10 wild type mice (EGFP vectors) or into Cx47 knockout mice (GJC2 vectors) either by L5-6 lumbar intrathecal injection (total of 2x10^11 vg in 20 µl) or by tail vein intravenous injection (4x10^11 vg in a 30µl volume) at a slow rate of 5µl/min. We examined EGFP and Cx47 expression 4 weeks after vector delivery by double immunostaining with relevant cell markers and by immunoblot analysis. We found that expression with both serotypes was restricted to oligodendrocytes, and was not detected in neurons, astrocytes or microglia. AAV9 provided overall higher expression rates in oligodendrocytes compared to AAVrh10 vectors. Moreover, intravenous delivery resulted in a more widespread biodistribution and gene expression in the CNS while lumbar injection mainly targeted the spinal cord. After confirming the higher efficacy of intravenously delivered AAV9-*Mbp.GJC2* to replace Cx47 in oligodendrocytes, we proceeded with a therapeutic treatment trial in groups of Cx32/Cx47 double KO (dKO) mice, an authentic model of PMLD1, with severe early onset demyelination. Mice (n=10 per group) were treated at P10 either with the AAV9-*Mbp.GJC2* therapeutic vector or with the AAV9-*Mbp.EGFP* (mock vector) and outcomes including motor behavior and CNS pathological changes were compared at P30. We found significant improvements in motor performance measured by the foot slip test, rotarod test, and foot print test in treated compared to mock-treated dKO mice. Analysis of myelination in corpus callosum, internal capsule, anterior and posterior spinal cord white matter confirmed improved myelin density and reduced vacuolation in treated mice. Furthermore, analysis of inflammatory glia responses showed improvement in astrogliosis and microglia activation in the CNS of treated mice. Finally, survival was significantly prolonged in treated compared to mock-treated dKO animals. This project provides a proof of principle for a gene replacement approach to treat PMLD1, as well as potentially other leukodystrophy forms resulting from mutations in structural genes expressed by oligodendrocytes.

247 Gene Therapy for Mucolipidosis IV Using Novel CNS-Targeted AAV Capsid

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Mucolipidosis IV (MLIV) is a lysosomal disease resulting in severe psychomotor deficits and vision loss. It is caused by loss-of-function variants of *MCOLN1* gene that encodes the lysosomal transient receptor potential channel mucolipin1, or TRPML1. With no existing therapy, the unmet need in this disease is extremely high. Recently we showed that AAV-mediated CNS-targeted gene transfer of the human MCOLN1 rescued motor function and alleviated brain pathology in the MLIV mouse model. We found that intracerebroventricular administration of AAV9-MCOLN1 in neonatal MLIV mice significantly improved motor function, myelination and reduced lysosomal storage load. Despite being currently a vector of choice for CNS-directed applications, AAV9 has biodistribution limitations such as low transduction of brain and retina from either systemic or CSF-directed routes of administration and toxicity concerns such as dorsal root ganglion (DRG) degeneration with intra-thecal administration. To achieve optimal therapeutic outcomes in MLIV, broad brain biodistribution is required. Here we report preclinical efficacy in MLIV mouse model using systemic administration of a novel AAV9-derived vector with enhanced CNS tropism expressing human *MCOLN1*. Compared to AAV9, this vector demonstrates 5-50-fold enhancement in transduction of the CNS and retina in both mice and non-human primates and is therefore of high advantage to efficiently rescue both CNS dysfunction and vision loss in MLIV. Intravenous delivery of this vector expressing human MCOLN1 in symptomatic *Mcoln1*$^{-/-}$ mice resulted in broad peripheral and CNS biodistribution, showed dose-dependent improvement of motor function and significantly delayed time to paralysis as compared with vehicle-treated group. Evaluation of brain pathology showed significant reduction of microgliosis (CD68) and astrocytosis (GFAP) in the brains of vector-treated *Mcoln1*$^{-/-}$ animals. Evaluation of lysosomal pathology and myelination is currently ongoing. These data suggest a new systemic gene replacement therapy for patients with MLIV mediated by a highly translatable novel AAV vector.

248 Development of an *Ex Vivo* Gene Therapy for Frontotemporal Dementia (FTD)

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Frontotemporal Dementia (FTD, OMIM:607485) is a severe and fatal adult neurodegenerative condition devoid of any cure or specific approved treatment. About 20% of familial FTD cases are caused by pathogenic loss-of-function mutations in the progranulin gene (GRN). GRN is a secreted lysosomal protein that functions as neurotrophic factor and regulator of neuroinflammation. Raising GRN levels in the brain of FTD patients, and in particular in microglia, may result in therapeutic benefit. Hematopoietic Stem Cell - Gene Therapy (HSC-GT) based on the use of lentiviral vectors (LVV) for gene transfer offers the potential benefit of long-lasting delivery of robust GRN levels in the central nervous system (CNS) of FTD patients, potentially restoring physiological microglia function, modulating neuroinflammation and attenuating neuronal damage. We developed and tested therapeutic LVVs with novel inducible synthetic promoters able to safely deliver multiple copies of the human *GRN* cDNA in GRN knock-out (ko) cell models. Upon cell transduction we observed over-expression of the therapeutic protein which was then secreted and token up by target GRN ko cells. The most promising among these constructs have been tested *in vivo* at different transgene doses (vector copy number [VCN] of drug products ranging from 1 to 8 copies) in optimized transplant conditions enabling CNS-specific and selective engraftment of the transplanted cells and of their progeny. In particular, cohorts

of young myeloablated GRN-ko mice were intracerebroventricularly transplanted with HSCs derived from GRN-ko previously transduced with LVV expressing the GRN under control of a strong constitutive promoter or myeloid/specific inducible promoters. With our approach we showed that GRN-transduced murine HSCs retain their proliferative and clonogenic capacity, they are able to engraft and repopulate the brain myeloid compartment of FTD mice, and to locally deliver GRN already at 6 weeks from the transplant. Moreover, the delivery of GRN into the brain of recipient mice is proportional to the degree of brain engraftment, to the transgene dose and can be controlled in a regulated fashion reflecting the nature of the promoter that drives transgene expression. We are currently monitoring cohorts of transplanted GRN-ko mice to evaluate the safety, feasibility, and long-term efficacy of the therapeutic strategy by comparing two different promoters (strong constitutive vs inducible) and transplantation routes (intra-CNS vs systemic). The efficacy of this potential therapeutic approach is being evaluated both at the behavioral and pathological level.

AAV Vector Genome Biology and Engineering II

249 Mechanistic Evaluation of Liver-Specific Transgene Repression from AAV Vectors in Non-Human Primates and Minipigs

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The recent FDA approval of Hemgenix for treating hemophilia B has shown that adeno-associated viral (AAV) vector mediated, liver-directed Factor IX expression can provide therapeutic benefits for patients. Circulating Factor IX in serum necessary to prevent bleeding is in the nanograms per milliliter range, relatively low compared to what is required for other indications. Significant reduction of mRNA levels in liver of non-human primates (NHP) when compared to mouse liver following AAV delivery has been reported by us and others. Liver-directed expression of an anti-plasma Kallikrein antibody (anti-pKal Ab) packaged in an AAV8 vector was evaluated in mice and NHPs where we observed equivalent copies of vector DNA delivered to the liver, but a 1000 to 3000-fold reduction in transgene mRNA levels in the liver of NHP versus mouse. Here we further discuss our findings in NHPs as well as evaluation of minipigs as an alternate animal model to study liver-specific expression from AAV vectors. To evaluate the effect of different AAV capsids (AAV8, AAV9 and AAV6), promoters (ApoEhAAT, TBG, CAG, LMTP6, LMTP24), routes of administration [Intravenous and Intra-muscular (one or multiple dose sites)], and different antibody formats (IgG vs scFv-Fc) on the expression of anti-pKal antibody in the serum as well as the mRNA expression from the liver, we conducted additional NHP studies. None of the modifications we explored significantly improved the protein and mRNA expression from the NHP liver. Minipigs were evaluated for liver-specific expression of anti-pKal Ab. AAV8-ApoEhAAT-Anti-pKal-Ab vector was injected intravenously into 2-month-old minipigs and monitored for 4 weeks. The serum levels of the secreted anti-pKal Ab were measured at 14- and 28-days post AAV delivery. At the same vector dose per kilogram bodyweight, minipigs and NHPs had similar serum anti-pKal Ab levels at time points examined, which were a 1000-fold lower than the levels in mouse serum for the same vector and dose. A similar trend was observed in the liver transgene mRNA levels between the 3 species with equivalent number of vector copies in the liver of all three species. RNAscope evaluation of liver sections from these studies correlated with the PCR analysis showing no or limited mRNA detected in NHP and minipig hepatocytes in the presence of AAV vector DNA in the nucleus. Thus, minipig recapitulated the liver-specific mRNA repression we observed in NHP and can be used for further evaluation of AAV vector mediated expression from liver. To gain understanding of the possible mechanism(s) underlying this significant difference in transgene expression in the liver of different species, we applied single cell multiome ATAC plus gene expression sequencing on liver tissue from different species treated with AAV vector. This technique enabled simultaneous analysis of transgene expression, differential gene expression pattern and AAV genome accessibility. The molecular structure and complexity of episomal AAV DNA were evaluated for mouse and NHP liver treated with AAV vector. Plasmid-Safe ATP-Dependent DNase and a single cutter restriction enzyme digestion followed by ddPCR quantification was used to investigate the average number of AAV genomes per concatemer: 1-2 in NHP liver vs 2-3 in mouse liver. When put together, we expect all these studies to help us understand factors contributing to the repression of mRNA levels in the liver of NHPs and minipigs.

250 HICOS: High-Throughput Screening of Codon Optimized Sequences Using Protein Barcoding

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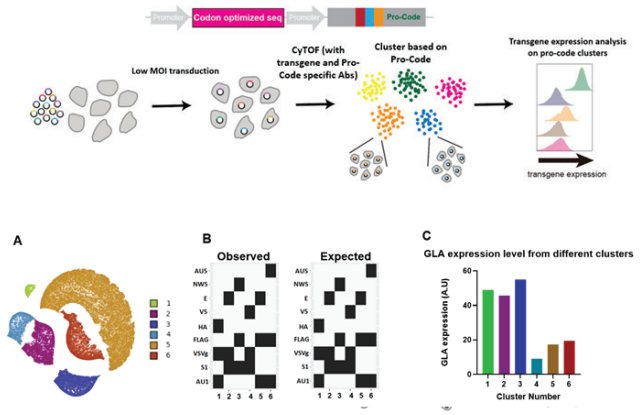
Introduction: Gene therapy using recombinant AAV (rAAV) vectors is a promising approach to treat many different genetic diseases. Despite its promise, one unmet challenge in gene therapy is the development of optimized transgene encoding sequences to achieve high levels of expression, while reducing vector immunogenicity. Higher vector doses have been associated with enhanced immune responses and, in some cases, systemic toxicities. Similarly, high content of CpG motifs, cryptic splice variants, and other sequence features can contribute to immunogenicity of AAV vectors. Codon optimization is a common approach to enhance expression levels while modulating vector genome-driven immunogenicity. However, a rate limiting step in the screening for top performing codon optimized cDNAs is the inability to screen large numbers of variants for protein expression *in vitro*. Here, we developed a protein barcoding system for high throughput codon-optimized screening (HICOS) to enable high-throughput screening of cDNAs (Figure 1). **Method:** Genetic barcodes detectable as proteins (Pro-Codes) are a cell barcoding system operating at protein level in which a fusion protein composed of different combinations of epitope tags (e.g., HA, V5, and FLAG) and dNGFR, a truncated receptor without an intracellular domain (Wroblewska *et al., Cell*

2018). Combinations of antibody-detectable epitopes form a higher order set of barcodes and Pro-Code expressing cells can be analyzed by time-of-flight mass cytometry (CyTOF). HICOS pairs Pro-Code and codon optimized cDNA to allow protein-level phenotyping in genetic screens. **Results: Figure 1.** Schematic of HICOS method. (Top) Dual promoter constructs were utilized to enable tandem expression of transgene and a unique protein barcode (Pro-Code) (Bottom) Cells are transduced with Pro-Code library using lentivirus at low MOIs (multiplicity of infection) such that each cell receives a single HICOS element. Following selection, a population of pooled transduced cells are stained with heavy metal conjugated antibodies and detected by CyTOF. Cells are clustered based on Pro-Code identity and linked to a quantitative measure of transgene expression. **Figure 2.** Stratification of codon-optimized GLA variants by HICOS. (A) HICOS assay comprised of 6 different CpG-depleted, codon-optimized GLA (alpha galactosidase A) variants (numbered 1 through 6) was conducted in Huh7 cells. Pro-Code clustering is based on epitope expression pattern and each cluster represents single CpG-depleted, codon-optimized GLA. (B) Unique combinatorial epitopes (Pro-Codes) mapped per cluster (C) Stratification of cDNAs into high-expressors (clusters 1-3) vs. low expressors (clusters 4-6). **Conclusion:** HICOS is a novel platform for efficient screening of codon optimized cDNAs. Our proof-of-principle study successfully identified the best performing variants in a set of codon-optimized cDNAs encoding the GLA protein. The HICOS approach may enable rapid identification of lead cDNAs encoding therapeutic transgenes.



251 Tissue Regeneration Enhancer Elements Enable Injury Responsive AAV Gene Expression

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Clinical translation of gene therapies for tissue regeneration will require precise spatiotemporal control of transgene expression to mitigate off-target effects, tissue overgrowth, and oncogenesis. Epigenetic profiling in regenerating tissues of zebrafish have revealed genetic enhancers that gain open chromatin marks after injury and during regeneration, thus termed tissue regeneration enhancer elements (TREEs). Recently, we reported these zebrafish TREEs can be modulated to drive gene expression in mammalian injury sites, both in mice and pigs. Here, we characterize TREE-driven AAV transgene expression in various mouse models of injury to map spatial and temporal dynamics. Specifically, we administered AAV vectors harboring TREEs upstream of an *Hsp68* minimal promoter and a firefly luciferase reporter transgene, intravenously to adult mice prior to injury induction. Injury models included cardiac ischemia-reperfusion, barium chloride muscle injury, and digit amputation. Injected mice were imaged with IVIS imaging in a longitudinal manner to track TREE-driven expression before and after injury. At the end of the imaging period, luciferase assays as well as biodistribution assays to quantify viral genomes in healthy and injured tissues were carried out. Taken together these results highlight on-target and off-target aspects of the transient and injury-site specific nature of TREE-driven expression in mice. Zebrafish TREEs are a promising new platform for inducing focal and transient gene expression at injury sites with potential clinical implications.

252 Strong Compact Neuron Specific Promoters for AAV Gene Transfer

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Recombinant adeno-associated viral (rAAV) vectors are currently considered the leading platform for *in vivo* gene therapy. However, the packaging capacity of AAV is limited to <5 kb, which excludes larger therapeutic genes from conventional vector designs. Many efforts have been developed to overcome this limitation, such as split rAAVs and fragmented rAAV strategies. A compact and efficient promoter will be beneficial for packaging and expressing larger transgenes. Here, we identified three compact promoters derived from the human survival motor neuron (*SMN1*) gene with lengths of 61 bp, 155 bp, and 303 bp. We constructed *EGFP* reporter plasmids harboring these three promoters and evaluated their activities in HEK293 and Neuro2a cells by plasmid transfection. All three promoters can drive efficient transgene expression in both cell lines, where the 155 bp promoter is the most efficient and the 61 bp promoter is the least. We then packaged these reporter constructs into self-complementary AAV9 (scAAV9) vectors and injected them into neonatal C57BL/6 mice at P1 at a dose of 4E11 vg/mouse by facial vein administration. An scAAV9 vector with the cytomegalovirus enhancer/chicken β-actin (*CMVen/CB)* promoter driving an EGFP reporter (scAAV-*CB/CMV-EGFP*) was injected side-by-side for comparison. After three weeks, we sacrificed the mice and examined GFP expression in brain, spinal cord, heart, muscle, and pancreas. We found that the 61-bp promoter exhibited neuron specific expression in the brain and spinal cord. However, no expression was detected in heart, muscle, or pancreas, suggesting that the 61-bp promoter is active in neurons of the central nervous system (CNS). Compared to the 61-bp promoter, both the 155-bp and 303-bp promoters showed stronger activities that were close to those achieved by *CB/CMV* promoter in the brain and spinal cord. Interestingly, the 155-bp and 303-bp promoters also showed heart activity that were comparable to those achieved by *CB/CMV*, but they lacked activity in skeletal muscle and pancreas. Further characterization of these promoters in adult mice is ongoing. In conclusion, our study has identified a small 61-bp promoter that is active in the neuron, and

two additional small promoters with lengths of 155 bp and 303 bp with activities in the neuron and cardiac cell.* **G.G., and J.X are co-corresponding authors**

253 Capsid Mediated Control of Adeno-Associated Viral Genome Transcription

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The host range and tissue tropism of Adeno-associated virus (AAV) is generally attributed to capsid-receptor interactions. While examining mammalian host cell permissivity to an Avian AAV isolate, we discover that AAV genome transcription is inhibited in a capsid-dependent manner. However, by swapping the VP1 N-terminus of primate-derived AAVs onto Avian AAV, we unlocked robust transduction. To determine why Avian AAV transcription is silenced in mammalian cells, we conducted a chromatin accessibility assay. While primate-derived AAV and an Avian/primate AAV chimera displayed chromatin accessibility resembling a euchromatin control locus, Avian AAV chromatin accessibility mirrored a heterochromatin control. To further interrogate whether epigenetic modification may play a role in Avian AAV genome silencing, we carried out a CUT&RUN assay to profile histone methylation. Specifically, we targeted H3K4me3, a marker for active transcription, and H3K9me3, a marker for transcriptional repression. H3K4me3 and H3K9me3 modifications were found on both Avian AAV and Avian/primate chimeric AAV genomes; however, their respective profiles were significantly altered. Moreover, significantly less H3K4 modifications and H3K9 modifications were found across the Avian AAV genome compared to the Avian/primate AAV chimera. Based on these results, we conducted a proximity ligation based, BioID spatial interactome analysis to identify host factors that interact differentially with Avian and Primate AAV capsids, specifically their respective VP1 N-terminal domains. Gene ontology analysis of primate AAV N-terminal interactors yielded a number of significantly enriched terms related to transcription and RNA processing. Our results corroborate the notion that the VP1 N-terminus of primate AAVs have evolved to recruit host factors that exert influence on AAV genome transcription. Knockdown experiments of several candidates within this interactome significantly reduced primate AAV transduction. Thus, mammalian host factors, including those linked to innate immune pathways, interact with the VP1 N-terminus in a capsid-specific manner to modulate the epigenetic status and transcriptional silencing of AAV genomes. Taken together, we identify a minimal structural component of primate-derived AAVs that regulates vector genome transcription in mammalian cells. We further postulate a mechanistic basis for how AAV capsids restrict host range independent of receptor interactions.

254 DNA Damage Repair Pathways Have Opposing Effects on Concatenation and Expression from rAAV Vectors

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Recombinant adeno-associated viral vectors (rAAVs) deliver DNA to a wide range of tissue types, but safe application to a broad spectrum of diseases is limited by vector genome (VG) capacity and requirements for large doses. Overcoming these limitations requires an understanding of how VGs are processed and regulated in recipient cells. rAAV VGs concatenate in host nuclei and can form episomes which stably express transgenes, but the mechanisms governing these central processes in gene therapy are unclear. To identify host factors involved in VG processing and potential druggable targets for increasing transduction efficiency, we performed a genome-wide CRISPR knockout screen for negative regulators of split transgene reconstitution, which relies on concatenation to express a trans-splicing dual vector. Genes are highly enriched in chromatin architecture and a specific subset of DNA Damage Repair (DDR) pathways, including several genes not previously studied in the context of AAV biology. Here, we focus on cellular proteins involved in double-strand break (DSB) repair. Using a split *lacZ* dual vector reporter system, we observe a 4-fold increase in split *lacZ* expression in cells depleted of BRCA1, which is involved in Homology Directed Repair (HDR). Pharmacological inhibition of other HDR factors, such as Rad51, induce a 10-fold increase in split *lacZ* expression. Conversely, inhibition of Non-Homologous End Joining (NHEJ) enzymes decrease split *lacZ* transduction by 10-fold.To examine the mechanisms underlying these effects at individual VG resolution, we [HT1] generated VGs that include 64 *lacO* repeats, a minimal CMV promoter, and 24 MS2 stem loops. Transducing cell lines that express LacI-mNeonGreen and MCP-mCherry enables visualization of rAAV VGs (mNeonGreen foci) and their nascent transcription (mCherry foci) *in-situ*. In addition to super-resolution imaging, we perform high content imaging to quantitatively assess VGs, expression, and colocalizing host factors in tens of thousands of cells per experiment. Compared to control cells, BRCA1 depletion or Rad51 inhibition increases the number and size of VG foci per cell in addition to the proportion of transcriptionally active VGs. Conversely, inhibition of NHEJ enzymes has the opposite effect. Immunostaining for cellular DDR factors reveals competitive occupancy of HDR and NHEJ factors on VG foci, with one dominating when the other is inhibited. We also observe colocalization of VGs with phosphorylated Histone 2 variant H2AX (γH2AX), the marker of chromosomal DSBs, as early as 2 hours post-transduction. Furthermore, inhibition of ATM kinase, which orchestrates DSB repair via γH2AX phosphorylation, drastically reduces the proportion of cells with VG foci, number of foci per cell, and average size of VG foci. Taken together, our observations suggest a model wherein VGs uncoat, quickly associate with histones, and are recognized in a similar manner to chromosomal DSBs most robustly by HDR factors. However, NHEJ may function more efficiently in concatenation and processing that

leads to transgene expression. Ongoing animal studies are testing the effects of HDR inhibition on dual vector transduction *in vivo*. Our novel findings provide insights into rAAV genome processing mechanisms and highlight how host factors can be manipulated to increase rAAV transduction, particularly for trans-splicing dual vectors that can express large transgenes for therapeutic applications.

255 Should You Judge an AAV by Its Cover? The Role the AAV Capsid Plays in Setting up the Vector Epigenome

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Adeno-associated viral vector (AAV) is the most commonly used vector for *in vivo* based gene therapeutics. However, the unpredictable discordance in vector mediated transduction between species poses a major challenge to selecting the optimal vectors for human clinical trials. Recombinant AAV-LK03, a capsid currently evaluated in several clinical trials was shown to robustly transduce human but not mouse hepatocytes in a humanized mouse model. To our surprise, a single-amino acid insertion, 266iG named AM, into the AAV-LK03 capsid managed to enable efficient transduction in mice while further enhancing transduction efficiency in human cells compared to AAV-LK03. Using Cut and Tag methods, we demonstrated that the limitation to transduction in mouse was unrelated to cell entry and nuclear transport but rather due to depleted histone H3 epigenetic modifications related to active transcription, namely H3K4me3 and H3K27ac, on the vector itself. We recently found that insertion of the 266G amino acid into a second primate selective capsid, AAV-NP59 increased transduction measured by transgene expression by ~30 fold in mouse cells. To identify which of the VP proteins is responsible for the dual tropism phenotype of AM, we created AAV-AM/LK03 VP hybrids as well as AAV-AM- and LK03- VP3 only virions. Our preliminary data suggest the 266iG in VP3 is primarily responsible for the observed dual tropism of the AM vector. Overall, these results suggest that the capsid proteins uncoat and the viral genome in the nucleus directly interact with host proteins to affect the epigenetic state of the viral episomal genome and that these interactions differ between species. To identify host factors that interact with the capsid and/or vector genome and are involved in this process, we performed anti-A20 immunoprecipitation and mass spectometry, followed by evaluation of candidate proteins on LK03 and AM transduction efficiency with siRNA silencing. Of note, our siRNA screen identified 3 novel proteins as host restriction factors critical for AAV transduction and we are establishing if and how these proteins might participate in setting up the vector epigenome. In addition, we are currently performing intranuclear AAV injections and fluorescence imaging to study potential spatial differences in nuclear localization between AAV-AM and AAV-LK03 delivered genomes and their effect on transgene expression. Unraveling the mechanism behind the observed differences in capsid-mediated vector epigenome dependence based on species will not only improve our understanding of basic AAV biology but also improve our ability to correlate outcomes in humans based on preclinical testing in animals.

Genome & Epigenome Editing Technologies I

256 A Metagenomics-Derived Gene-Editing Toolbox Enables Efficient Genome Engineering with Nucleases and Base-Editors in Primary Cells and *In Vivo*

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Gene-editing technology has revolutionized molecular therapeutics, enabling engineering-based approaches to treat disease. Despite this, development of medicines using gene editing has been hampered by technological, immunological, and legal limitations. We described previously the discovery of two novel gene-editing systems from metagenomic data, the characterization of these systems in vitro, and a demonstration of their activity. Here, we add multiple additional nucleases from metagenomic sampling so as to create a genome-editing platform capable of targeting almost every base pair in the human genome. The platform is composed of a "menu" of both type II and type V nucleases, capturing the PAM diversity of the former and the increased specificity of the latter. Our metagenomic approach discovers entire families of editing systems rather than isolated enzymes. We take opportunistic advantage of the structural conservation and PAM diversity across these families to perform PAM-interacting domain (PID) swaps between family members, substituting PIDs from less-active members into the backbone of a more-highly active nuclease. Such chimeric enzymes rapidly and significantly increase the targeting density of the platform. Further, we engineer one of our nucleases into an adenine base editor (ABE), sourcing the adenine deaminase from our metagenomic database. We achieve up to 95% A-to-G conversion in cultured cells. The ABE was engineered in the context of one of our chimeric nucleases, allowing our base-editing platform to share the high targetability conferred by our PAM library. Finally, as our gene-editing systems are sourced from microbes found in environmental samples and from the non-pathogenic human microbiome, we expected that pre-existing immunity to our nucleases would be quite rare. We demonstrate this is indeed the case, as there is no detectable pre-existing antibody immunity in a panel of serum samples from forty-eight randomly selected donors. In all, we show

that these new gene-editing systems have the activity, specificity, and translatability necessary for broad use in genetic medicine development.

257 Programmable A-to-Y Base Editing by Fusing an Adenine Base Editor with an N-methylpurine DNA Glycosylase

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Background: Base editors are promising tools for precise base-editing in basic research and therapeutic applications. For years, adenine base-editors (ABEs), cytosine base-editors (CBEs), and C-to-G base-editors (CGBEs) have been developed and widely used. However, base editor enabling A-to-T and A-to-C transversions remains to be achieved to repair large number of point mutations, accounting for up to 27% genetic diseases. **Methods**: To induce A-to-T and A-to-C transversion editing, we hypothesized that excision of ABE-induced deoxyinosine might enable more versatile base-editing outcomes by triggering the base-excision-repair (BER) pathway in cells. We developed three prototype versions of adenine transversion base-editor (AYBE, Y = C or T base) by fusing ABE8e to wild-type human N-methylpurine DNA glycosylase protein (MPG), which could excise hypoxanthine in damaged DNA at different orientations with respect to nCas9. An intron-split EGFP reporter system with an inactive splicing acceptor signal was engineered to evaluate the transversion activity of AYBE by introducing disruptive point mutations into the intron boundary. Only A-to-T or A-to-C transversion could correct the mutation for proper splicing of EGFP-coding sequence, thus activating EGFP expression. To improve AYBE activity, we performed rational mutagenesis of MPG and generated hundreds of AYBE variants for screening (Fig. 1A). A proof-of-concept study using a stable HEK293T cell line generated via lentiviral transduction was performed to investigate the therapeutic potential of AYBE variants for correcting disease-related transversion mutations. Finally, we further increase the efficiency and purity of A-to-T editing outcomes by engineering ATBE. **Results**: We detected 56.6% and 7.32% of EGFP$^+$ cells using AYBEv0.1 (with wild-type MPG fused at the C-terminus) with A-to-T and A-to-C reporter, respectively. After rounds of mutagenesis screening, we found synergistic enhancement of transversion editing activity by 4.78-fold for AYBEv3 variant in comparison with AYBEv0.1 (Fig. 1B). AYBEv3 also exhibited efficient A-to-Y transversion editing activity when replaced nCas9(D10A) with dCas9 or dCas12. Furthermore, the engineered AYBEv3 variant could enable targeted editing with high transversion editing activity (up to 72% for A-to-C or A-to-T editing). We also found approximately 36% and 44% correction frequencies of A-to-C edits at *DMD* and *SLC26A4* nonsense mutation sites and approximately 11% and 20% correction frequencies of A-to-T edits at *ATM* and *TTN* splicing acceptor site mutations in the stable lentivirus-transduced HEK293T cells, respectively. The engineered ATBE could mediate A-to-T transversion efficiency up to 52% and the purity up to 79% (Fig. 1C). **Conclusions**: Our findings with engineering a novel AYBE for effective A-to-T and A-to-C editing provide the complementary toolkit (Fig. 1D) to the current base-editor repertoire for modeling and treating disease-causing transversion mutations in humans. A more accurate ATBE would enhance the therapeutic potential of the base-editor platform.

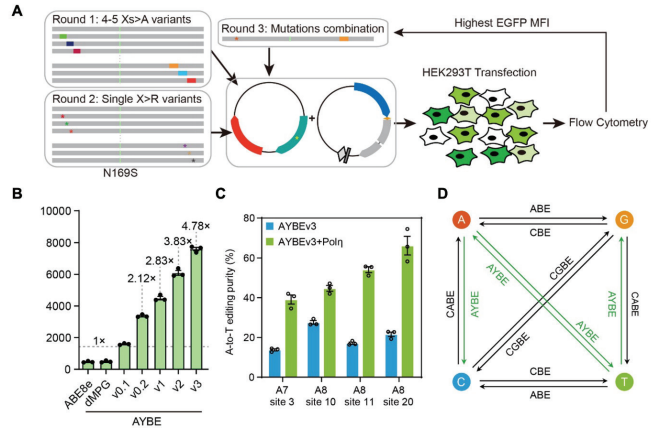

Fig. 1. Engineering and optimization of adenine transversion base editor. (**A**) Schematic of mutagenesis and screening strategy. (**B**) Gradual improvement of AYBE-mediated EGFP activation. Dotted line, mean value of wild-type MPG group. Fold changes are calculated relative to the wild-type MPG group. (**C**) A-to-T editing outcomes for the introduction of Polη. (**D**) Diagram showing types of achievable point mutations with the available base editors. n = 3, all values are presented as mean ± s.e.m.

258 Multiplexed Epigenome Editing to Induce Sustained Silencing of Immune Checkpoints' Expression in CAR T Cells

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The development of an exhausted phenotype is one of the major obstacles to the success of chimeric antigen receptor (CAR) T cell therapy. Amongst the hallmarks of T cell exhaustion, one of the best characterized is the overexpression of immune checkpoint receptors, such as PD-1, CTLA-4, LAG-3, TIM-3 and others. When these receptors encounter their respective ligands, expressed from either tumor cells or other components of the tumor microenvironment, activation of inhibitory intracellular pathways impairs T cell functions. Therefore, interrupting such interaction became an attractive strategy to protect T cells from exhaustion. Immune checkpoint blockers have shown promising results in this regard, reinvigorating the immune response against tumors. Moreover, combined blockage of multiple immune checkpoints elicits synergistic effects. However, the occurrence of severe immune-related side effects due to the systemic activity of the blockers, and the adaptation of the tumor microenvironment that can eventually lead to resistance, are still unresolved. We have devised a strategy to render CAR T cells devoid of two major immune checkpoints via hit-and-run epigenome editing. Designer epigenome modifiers (DEMs) targeting *PDCD1* or *LAG3* were delivered by electroporation, in the form of in vitro transcribed mRNA in activated T cells expressing a 2nd generation CAR capable of specifically recognize the prostate-specific membrane antigen (PSMA), an antigen highly expressed on prostate cancer cells. This one-time intervention led to the deposition of de novo

cytosine methylation in their respective promoters, sustainably and simultaneously inducing silencing of the two genes in the targeted cell population. The epigenetically induced silencing was persistent throughout repetitive CAR T cell activation through direct contact with PSMA-positive target cells. Furthermore, the epigenetically edited CAR T cells were fully functional and indistinguishable from their parental unmodified cells in an array of in vitro assays including cytotoxic capacity, cytokines release, differentiation towards effector phenotype and proliferative ability, thus, demonstrating the safety of DEM-mediated epigenome editing in CAR T cells. The specificity of these DEMs to target *PDCD1* and *LAG3* is currently being assessed. However, DEMs targeting other loci have shown remarkable conserved specificity, encouraging the anticipation of favorable outcome. Our results propose this technology as a novel approach to modulate diverse T cells inhibitory pathways needless of DNA sequence modification, in a simultaneous and targeted manner employing a protocol compatible with the current clinical application of CAR T cell therapy.

259 Novel Cas9 Orthologs Expand the Genome Editing Toolbox for CRISPR-Based Therapeutics

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Genome editing holds great promise for therapeutic applications, however its full potential is hindered by the current limitations of the genome editing toolbox, such as delivery, safety and PAM requirements. One way to overcome these limitations is to expand the toolbox by identifying novel Cas9 proteins with unique properties. In this work, we interrogated a massively expanded microbiome dataset (above 1 million bacterial genomes, >90000 Cas9 loci) to identify novel Cas9 proteins characterized by desirable features, including reduced molecular size (<1100 aa), variegated phylogenetic distribution, low homology to previously characterized nucleases, and diverse PAM specificities, as determined by *in silico* prediction. Among the Cas9 orthologs carefully selected for validation, most of them were shown to be active in mammalian cells. Notably, we identified 4 candidates showing comparable editing activity to the widely used SpCas9, reaching more than 90% EGFP knock-out in reporter cells. The activity of these nucleases was further compared to other widely used Cas9 proteins, showing comparable editing levels on endogenous loci and improved specificity, as determined by GUIDE-seq. Furthermore, given their reduced molecular size, these newly identified Cas9 proteins, as opposed to SpCas9, could be packaged with their sgRNA within a single all-in-one AAV vector, either as active nucleases (>40% editing) or nickases fused to an adenine-base editor (>60% A-to-G conversion). Finally, we also explored their potential for the development of *in-vivo* AAV-based gene editing approaches for the treatment of inherited retinal diseases and *ex-vivo* applications. The identification of these novel Cas9 proteins, with their small size, high activity and diverse PAM recognition sequences expands the genome editing toolbox and provides new avenues for the development of safe and effective therapies for a broad range of diseases.

260 Multiplexed Editing without Chromosomal Rearrangements Using Epigenetic Editors

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The ability to simultaneously alter the expression of multiple genes is desirable for many genome editing applications, such as optimizing CAR T cell function. However, conventional genome editing technologies that rely on double stranded DNA breaks (DSBs) have the potential for undesirable consequences beyond the generation of insertions and deletions (indels) at the target DSB sites, such as large deletions, translocations, and even chromothripsis. Genome editing many target genes leads to more DSBs thereby increasing the potential for such events. Epigenetic editing, in contrast, enables durable modulation of gene expression without cutting, nicking, adding to, deleting, or altering the DNA sequence. This makes epigenetic editing extremely well-suited for simultaneous targeting of multiple genes. In this study, we performed simultaneous silencing of three target genes in primary human healthy donor derived T cells using CRISPR-Off, a dCas9-based epigenetic editor. We compared CRISPR-Off silenced cells to cells edited at the same genes using Cas9 and evaluated chromosomal rearrangements using: (1) a quantitative sequencing-based assay that measures both small indels and structural rearrangements at guide RNA target sites; and (2) a fluorescence-based technique with single-cell resolution that can visualize structural rearrangements at the target sites. As expected, indels, which result from error prone repair of DSBs were only observed in the Cas9-treated cells and not in the CRISPR-Off treated cells. In addition, and in agreement with published literature, cells that had undergone multiplexed gene editing using Cas9 also displayed genomic rearrangements including translocations and truncations. In contrast, the rates of indels, translocations, and other chromosomal rearrangements in CRISPR-Off treated cells were similar to the background levels in untreated controls, while achieving knockdown of expression of all three genes. These data therefore reveal an important advantage of epigenetic editing compared to Cas9 genome editing in allowing genotoxicity-free multiplexed gene editing.

261 Metagenomic Mining and Engineering of Single-Strand Annealing Proteins for Cleavage-Free Genomic Knock-In of Long Sequence in Mouse Hepatocytes via dCas9 Targeting

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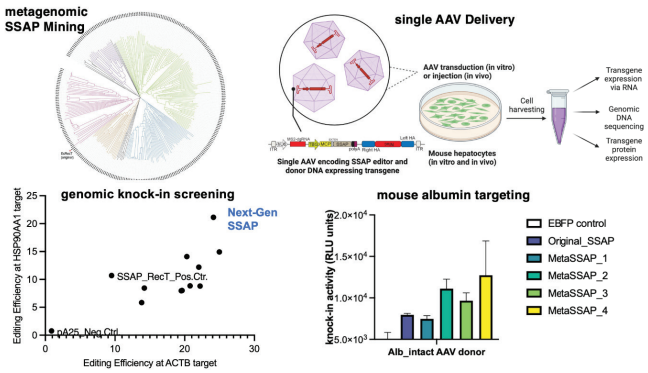
INTRODUCTION The ability to edit long sequences with high accuracy and minimal errors is a highly sought-after technology in gene-editing. Such a tool has potential for diverse research areas and applications, from generating knock-in models, to engineering

therapeutic transgene for gene and cell therapy. New waves of editors have facilitated genome modifications at different scales, but the efficient, precise insertion of long sequences (kilobase-scale knock-in) in primary, post-mitotic cells is still challenging. RESULTS Here, building on our prior demonstration of knock-in editing with dCas9 coupled to phage single-strand annealing protein (SSAP), we performed a metagenomic mining and genomic knock-in screening of SSAP editors in human cells. We identified several high-activity candidate SSAPs with close to ~3-fold improved editing efficiencies compared with our previous SSAP benchmarks. Top candidate achieves over 40% knock-in efficiency without any selection. We show that the newly engineered cleavage-free SSAP editor efficiently inserts kilobase-scale sequences at endogenous Albumin locus in primary mouse hepatocytes. To achieve this, we integrated our engineered metagenomic SSAP (metaSSAP) editors with AAV-based delivery. Importantly, thanks to the compact size (200-300 amino acids) of metaSSAPs, we could fit the entire SSAP editor and the transgene cargo within a single AAV vector. Further, longitudinal expression assay and insertion mapping demonstrate the genomic insertion of transgene by SSAP is durable and accurate in primary hepatocytes. CONCLUSION We developed a next-generation dCas9-SSAP editor using novel metagenomic SSAPs. This editor has high knock-in efficiency and accuracy in challenging primary hepatocytes settings. Thus, this showed that the SSAP recombination approach can mediate gene insertion in post-mitotic cells. Our work leads to a new option for large knock-in for in vivo scientific discovery and therapeutic development via recombination-mediated gene-editing. FIGURE 1: Mining and development of metagenomic SSAP for recombination-based genomic insertion of long sequences in hepatocytes.



*This abstract, Wang et al. entitled, "Metagenomic mining and engineering of single-strand annealing proteins for cleavage-free genomic knock-in of long sequence in mouse hepatocytes via dCas9 targeting", is submitted in accordance with ASGCT policy and with the understanding that it is kept under embargo and hence confidential until April 17, 2023.

262 Comprehensive Engineering of CasX Holoenzyme to Create an X-Editor with High Activity, Specificity and Deliverability

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The use of RNA-guided endonucleases from CRISPR-Cas systems has revolutionized the cell and gene therapy field. The need for Cas enzymes with reduced size, increased specificity, or distinct editing profiles or PAM preferences has motivated the identification and characterization of diverse novel Cas effectors. However, many of these naturally occurring effectors have poor activity in mammalian cells. Here, we present our efforts to engineer the CasX (Cas12e) holoenzyme for improved activity. CasX, like Cas9 or Cas12a, is a Class 2 Cas effector enzyme that binds and cuts dsDNA substrates when loaded with a matching guide RNA. Unlike the most active Cas9 and Cas12a variants, however, CasX is small enough (<1000 amino acids) to fit in an AAV vector with multiple guides or complex regulatory elements, generates distinct deletion patterns, and has less tolerance for mismatches between the guide RNA and dsDNA substrate. By building comprehensive and holistic libraries containing tens of thousands of distinct CasX molecules and screening them in both bacterial and human cell systems, we were able to identify mutations that increase the stability, DNA-binding ability, and cleavage activity of the protein. We then applied deep mutational scanning and selection methods to generate protein variants with broadened or respecified PAM preferences, thereby expanding genome targetability. A combination of high-throughput pooled screening and rational design was applied to the CasX sgRNA to yield a guide with significantly improved performance in both transcription-based and fully in vitro systems. The resulting improved "X-Editor" (XE) ribonucleoprotein (RNP) exhibits editing activity orders of magnitude higher than wild-type CasX molecules. We characterized XE editing efficiency compared to other engineered small Cas systems across dozens of loci in human cells, and further show that XE can achieve knock-out rates of immuno-oncology targets equal to or better than SpyCas9 when delivered as RNP to primary human immune cells. We demonstrate that XE is a potent and flexible platform for gene editing, with high activity, small size, and a range of accessible PAMs allowing it to be used across different delivery modalities and targets.

CAR Immunotherapy

263 Epitope Engineered Hematopoietic Stem and Progenitor Cells to Enable Multi-Specificity CAR-T Cell Immunotherapy for Acute Myeloid Leukemia

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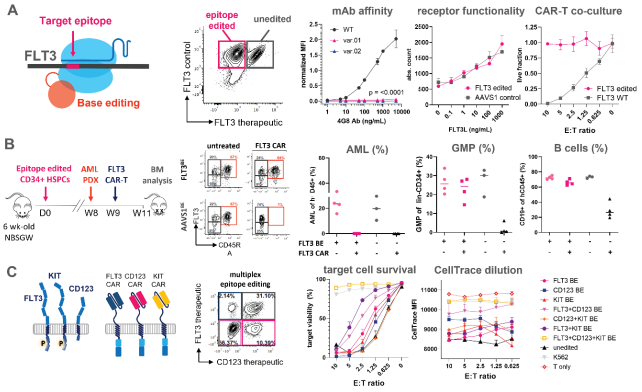
Despite treatment advances and the use of allogeneic hematopoietic stem/progenitor (HSPC) cell transplantation (HSCT), acute myeloid leukemia (AML) is associated with an unfavorable outcome for >50% of patients. While immunotherapies have proved compelling efficacy when targeting a dispensable lineage antigen (Ag), such as CD19 in B-ALL, their application for AML is hampered by the absence of actionable leukemia-restricted targets. Suitable targets are shared with healthy progenitor or mature myeloid cells, leading to on-target off-tumor toxicity. For these reasons, AML immunotherapies are typically restricted to a narrow time window before HSCT, reducing the chances of AML eradication. We found that specific epitopes on essential tumor-associated genes can be base-edited in healthy HSPCs to avoid the recognition by therapeutic Abs while preserving physiologic protein expression, regulation, and function. Precise epitope engineering allows targeting genes required for leukemia survival regardless of functional role in HSPC, minimizing the risk of immune escape. To this end, we selected the cytokine receptors *FLT3*, KIT and CD123, which are found in >85% of AML cases and whose mutation or overexpression is associated with poor prognosis. We identified single amino acid substitutions in FLT3, KIT and CD123 extracellular-domains that abrogate recognition by a therapeutic mAb while preserving physiologic surface expression, ligand-binding, kinase activation and signal transduction. These variants were resistant to CAR killing and did not induce CAR activation during in vitro co-culture (FIG.A). We identified a set of gRNAs that enable introduction of these mutations by adenine base editors (BE) without the need for dsDNA breaks. Electroporation of ABE8e mRNA and gRNAs into CD34+ HSPCs achieved up to 90%, 85% and 75% editing efficiency on FLT3, KIT and CD123 genes, respectively, either as single or multiplex. To assess if CAR-T cells can effectively eliminate AML while sparing the edited hematopoiesis, we sequentially engrafted NBSGW mice with edited HSPCs, human patient derived AML xenografts (PDX) and FLT3 or CD123 CAR T cells. FLT3 and CD123 edited HSPC sustained long-term multilineage hematopoiesis indicating successful editing of HSCs. Upon treatment with CAR-T, we observed eradication of AML PDX cells while sparing healthy hematopoiesis (B cells, GMP, LMPP and CD34+38- HSPCs for FLT3-CAR; dendritic cells and myeloid lineages for CD123-CAR) in mice engrafted with edited HSPCs but not in $AAVS1^{BE}$ controls (FIG.B). Epitope editing can be multiplexed to enable multi-specific CAR-T cells, with combinations of FLT3, KIT and CD123 providing superior protection when challenged with triple-specific CAR-T in vitro (FIG.C). This enabled multi-target immunotherapies that could eradicate PDX that were resistant to FLT3 CAR monotherapy in vivo. In conclusion, transplantation of epitope engineered HSPCs endowed with selective resistance to CAR-T or Abs is a novel approach that can be rapidly implemented in currently used HSCT protocols to enable more effective and safer immunotherapies for high-risk AML patients.



264 IL-12 Reprograms CAR-Expressing NKT Cells to Long-Lived Th1-Polarized Antitumor Effector Cells

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Natural Killer T cells (NKTs) are characterized by the expression of an invariant TCR (iTCR) that recognizes glycolipids, presented in the monomorphic CD1d molecule. The advantages of NKTs are the lower alloreactive potential, easier generation as off-the-shelf product, and ability to infiltrate solid tumors. Heczey A. et al. in 2014 showed the potential of GD2 CAR NKT cells in eliminating neuroblastoma (NB) tumor cells, although the tumor would eventually relapse. We propose to further engineer GD2.CAR NKTs to produce Interleukin 12 (IL-12). IL-12 is a heterodimeric cytokine composed of p35 and p40 chains with proinflammatory and immunoregulatory properties. The transduction efficiency of the NKTs evaluated by measuring the expression of GFP or CAR was superior to 70%. IL-12 was produced by IL12(I)GFP and GD2.CAR(I)IL2 NKTs and was detected in the supernatant (3564±236 pg/ml and 1661±1439 pg/ml respectively). To validate IL-12 pro-inflammatory effects, we analyzed the production of IFNγand IL-4 by NKTs upon stimulation through the iTCR. IL12(I) GFP and GD2.CAR(I)IL12 NKTs showed superior release of IFNγ and reduced release of IL-4 compared to control NKTs (7785±1661 pg/ml, 8807±3328 pg/ml and 7785±2477 pg/ml of IFNγ respectively and 238±117 pg/ml, 46±28 pg/ml, 587±213 pg/ml of IL-4 respectively). IL12(I)GFP NKTs showed increased proliferative capacity upon

activation through the iTCR and higher expression of CD62L compared to GFP NKTs (82±11% vs 39±11% respectively), which is consistent with our previous observation that CD62L expression in human NKTs identifies a cell subset with a high proliferative capacity. IL12(I)GFP NKTs showed superior expansion and persistence in vivo in peripheral blood (P<0.0001), spleen (P=0.0059), and liver (P=0.0023) of NSG mice up to day 30, and more than 50% of NKTs maintained CD62L expression. At day 30, IL12(I)GFP NKTs were selected from the spleen and injected in new non-tumor-bearing NSG mice to mimic a serial transplant. IL12(I)GFP NKTs showed consistent engraftment in the serial transplant and maintained CD62L expression. Importantly, mice infused with IL12(I)GFP NKTs did not show signs of graft versus host disease. To measure the antitumor activity of GD2.CAR(I)IL12 NKTs, we used NSG mice engrafted with the NB cell line CHLA-255. By day 63 all mice treated with GD2.CAR NKTs developed tumor, while mice treated with GD2.CAR(I)IL12 NKTs remained tumor-free. GD2.CAR(I)IL12 NKTs continued to protect the mice from tumor growth after tumor rechallenge and promoted prolonged tumor-free survival (P=0.0004). Only GD2.CAR(I)IL12 NKTs were detectable in the peripheral blood and in the liver and spleen at the time of euthanasia while maintaining CD62L expression.The advantages of IL-12 in NKTs, were also validated in combination with anti-CD19 CAR in leukemia and lymphoma, and anti-CSPG4.CAR in melanoma and osteosarcoma in vitro models.Overall, these data show the advantages of engineering NKTs to co-express a CAR and IL-12. Transgenic IL-12 initiates a transcriptional program in NKTs that leads to the generation of NKTs characterized by a high potential for in vivo expansion, persistence, and superior anti-tumor activity which can have a significant impact for adoptive NKT cell-based therapies.

265 Knocking-Down Expression of BTG1, a Key Driver of NKT Cell Exhaustion, Promotes Durable Tumor Control by CAR-NKTs in a Xenogeneic Neuroblastoma Model

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After infusion into cancer patients, T and NKT cells expressing tumor-specific chimeric antigen receptors (CAR) often become hyporesponsive or exhausted, limiting their antitumor potential. However, the mechanisms underlying this hyporesponsiveness remain poorly defined. As part of our ongoing phase 1 evaluation of autologous NKTs co-expressing a GD2-specific CAR with interleukin (IL)15 (GD2-CAR.15) in children with neuroblastoma (NB)(NCT03294954), we performed single-cell RNA sequencing of both infusion products and infused NKTs derived from the peripheral blood of 12 patients at 2-3 weeks post-infusion. We identified B cell translocation gene 1 (*BTG1*) as a top upregulated gene in GD2-CAR.15 NKTs isolated from patient peripheral blood (**Fig 1A**). Consistent with the known role of BTG1 in maintaining murine naïve T cell quiescence, we found that unstimulated human NKTs and freshly isolated naïve T cells downregulate BTG1 expression shortly after TCR activation. However, continuous TCR stimulation led to re-expression and sustained upregulation of *BTG1* in NKTs and T cells. In gain-of-function studies, NKTs transduced with a BTG1 construct (OE) showed a decrease in global RNA content (**Fig 1B**) as well as decreased expression of genes associated with the TCR, cytokine signaling, and AP1 transcription factor activity. Functionally, NKTs overexpressing *BTG1* demonstrated an inhibited response to TCR stimulation ($p < 0.001$) due to increased cell death and markedly reduced proliferation compared to control NKTs (**Fig 1C-F**). To determine whether *BTG1* knockdown (KD) improves the efficacy of CAR-NKT immunotherapy, we generated a set of novel constructs that encode the GD2-CAR only or the GD2-CAR with IL15 (used in our clinical trial), each with and without an artificial microRNA targeting *BTG1* (CAR.15.amiR-BTG1) or a scrambled microRNA control (CAR.15.amiR-SCR). Compared to CAR.15.amiR-SCR NKTs, we found that CAR.15.amiR-BTG1 NKTs are enriched for CD62L+ central memory-like cells, express lower levels of PD1, mediate enhanced short- and long-term cytolytic activity, and undergo a greater degree of expansion throughout a serial tumor challenge assay (**Fig 2A-F**). Additionally, compared to control groups, CAR.15.amiR-BTG1 NKTs mediated durable antitumor activity in an aggressive model of metastatic neuroblastoma, inducing complete tumor regression in all treated animals (**Fig 2G-I**). These results establish BTG1 as a novel mediator of hyporesponsiveness in human NKT and non-naïve T cells and suggest that BTG1 silencing may broadly benefit the development of cancer immunotherapy.

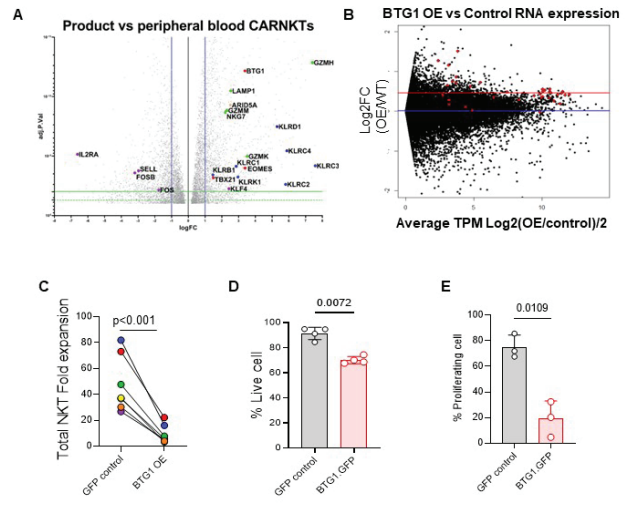

Figure 1. BTG1 expression is associated with CAR-NKT hyporesponsiveness. A. Volcano plot showing differentially expressed genes in pre-infusion product versus peripheral blood CAR-NKTs in patients treated with GD2-CAR NKTs. **B.** Total RNAseq reads from BTG1-overexpressing (OE) and GFP control NKTs. Red and blue lines indicate fold change from nuclear or mitochondrial transcripts, respectively. **C.** Fold expansion of BTG1-OE vs control NKTs after transduction. **D, E.** Survival of NKTs stimulated with aCD3/CD28 monoclonal antibodies and evaluated with annexin-V and fixable viability dye e780 on day 3, (**D**) and CellTrace Violet dilution measured on day 5 (**E**.).

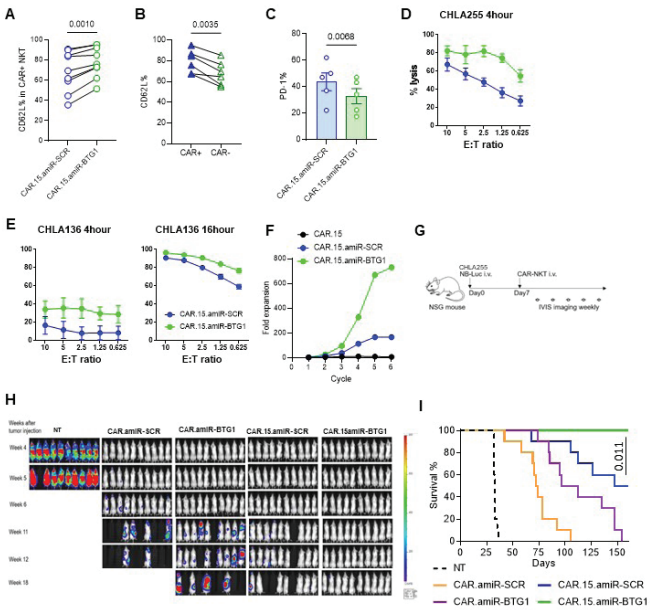

Figure 2. BTG1 knockdown (KD) enhances GD2-CAR.15 NKT antitumor activity. NKTs were transduced with retroviral vectors encoding the GD2-CAR with and without IL15 and artificial microRNA (amiR) specific for BTG1 or a scrambled control. **A.** CD62L expression in CAR-NKTs transduced with indicated constructs. **B**. CD62L expression in CAR.15.amiR-BTG1 NKTs in CAR+ versus CAR- populations. **C**. PD-1 expression in CAR-NKTs transduced with indicated constructs. **E**. Cytotoxicity of CAR.15.amiR.BTG1 (green lines) and scrambled control NKTs (blue lines) against GD2-high CHLA255 and GD2-low CHLA136 NB cell lines assessed at indicated co-culture time-points. **F**. Fold expansion of indicated CAR-NKT groups over the course of six co-culture cycles with CHLA255 cells. **G**. *In vivo* CAR-NKT antitumor activity experimental design using an aggressive metastatic neuroblastoma xenograft model. **H**. Bioluminescence images of neuroblastoma tumor-bearing mice injected with indicated NKT groups at specified timepoints. **L**. Kaplan-Meier survival curves for mice in indicated groups. 10 mice per therapeutic group, survival comparison by Gehan-Breslow-Wilcoxon test.

266 Elucidating the Role of PD-1/PD-L1 Axis in Solid Tumour CAR-T Cell Therapy

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Introduction Dysfunction of therapeutic T cells can be a major challenge for CAR-T cell therapy against solid tumors. One possible strategy to overcome this situation is the inhibition of the PD-1/PD-L1 axis by genetic engineering. However, there is still controversy over whether chronic PD-1 inhibition can prevent or accelerate CAR-T cell exhaustion. Here, we explore the effects of PD-1 deletion on CAR-T cell function by testing different CAR constructs in a tumor model expressing varying PD-L1 levels. **Methodology** To generate a tumor model of PD-L1 expression, we eliminated *CD274* from ovarian cancer cells (SKOV3) by CRISPR-Cas9. PD-L1 negative cells were then sorted and transduced with lentiviral vectors encoding PD-L1 under the control of constitutive promoters of low (PGK100), medium (PGK300) or high (EF1α) intensity. We confirmed that PD-L1 expression levels after IFNγ or CAR-T cell exposure were similar to commercial cancer cell lines by flow cytometry. For CAR-T cell generation, T cells were genetically engineered to express CARs targeting HER2 either with low (LA-HER2) or high affinity (HA-HER2) or mesothelin (Meso) and containing the CD28 intracellular domain in tandem with CD3z. *PDCD1* was knocked out on CAR-T cells by using CRISPR-Cas9. The frequency of *PDCD1* ablation was between 50-90% in all normal donors, as determined by Sanger sequencing. T cell polyfunctionality was analyzed using IsoCode Single-Cell Adaptive Immune from Isoplexis and gene expression was analyzed using the CAR-T cell characterization panel, from Nanostring. **Results** LA-HER2 CAR-T cells were highly sensitive to the PD1-PDL1 axis inhibition, even when PD-L1 was expressed at low densities. In this setting, knocking out PD-1 restored cytokine secretion and increased T cell polyfunctionality. Transcriptomic analyses following antigen recognition revealed significant differences in the gene expression pattern of PD-1 KO CAR-T cells as compared to CAR-T cells, including an upregulation of signatures related to T-cell activation and Th1 polarization. *In vivo*, LA-HER2 CAR-T cells were able to induce complete responses (CR) in 87% of mice bearing tumors in the absence of PD-L1 expression (SKOV3 PD-L1 KO), while the percentages of CR in animals with PD-L1-expressing tumours (SKOV3 PD-L1 low, high or wild-type) were below 25%, with most of the animals showing progressive disease. By contrast, genetic *PDCD1* ablation strikingly enhanced tumour clearance by CAR-T cells, with more than 87% of animals with PD-L1-expressing tumours undergoing CR after treatment. We also confirmed a beneficial role of PD-1 inhibition in LA-HER2 CAR-T cells against other cancer types expressing HER2, as well as in meso-28z CAR-T cells both *in vitro* and *in vivo*. Surprisingly, in CAR-T cells expressing HA-HER2, *PDCD1* ablation led to milder increases in cytokine release, and no differentially expressed genes were detected between PD-1 KO CAR-T cells and CAR-T cells. Also, we did not observe an increase in antitumor efficacy after PD-1 deletion in any of the *in vivo* models tested, including tumors with high PD-L1 densities, in striking contrast to what we had observed in LA-HER2 CAR-T cells. **Conclusions** In summary, our results show that knocking out PD-1 improves the efficacy of CD28-based CAR-T cells (LA-HER2 and meso) both *in vitro* and *in vivo*. In contrast, this advantage was mitigated when using HA-HER2 CAR-T cells, suggesting that CAR affinity may be an important factor modulating the sensitivity to PD-1-PD-L1 axis inhibition in CAR-T cell therapy. We are currently investigating the mechanisms behind these differences. Importantly, knocking out PD-1 was not detrimental in any of the CAR constructs used, even when their sensitivities to PD-1-PD-L1 inhibition diverged.

267 Non-Virally Engineered Polyclonal γδ T Cells Exhibit Potent Anti-Tumor Activity *In Vivo*

Jacob Bridge, Patricia Claudio-Vazquez, Sophia Wenthe, Joshua Krueger, Branden S. Moriarity, Beau Webber

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Introduction: γδ T cells are a subset of lymphocytes that recognize non-peptide antigens via canonical γδ T cell receptors (TCRs). Due to their diverse functionality and MHC-independent cytolytic activity, γδ T cells have garnered enthusiasm as a potential allogeneic immunotherapy. However, their heterogeneity and infrequency within peripheral blood, combined with inefficient genetic engineering and expansion of populations bearing polyclonal γδ TCR repertoires, has limited their clinical application. Here we implement a novel procedure for the large-scale production of polyclonal, non-virally engineered CAR-γδ T cells possessing potent anti-cancer activity. *In vivo* tracking of CAR-γδ T cells reveals selective persistence of specific

γδ T cell subsets correlating with therapeutic efficacy, providing opportunities to further refine the composition of γδ T cell products. **Methods:** Primary human γδ T cells were isolated from peripheral blood via immunomagnetic separation, then stimulated using plate-bound pan-γδ TCR and soluble CD28 antibodies (Fig. 1A). Non-viral transposon-based integration of a CD19 chimeric antigen receptor (CAR) construct was employed to direct intrinsic cytolytic activity against target cancer cells *in vitro* and *in vivo*. Candidate genes linked to γδ T cell inhibition, including *CISH*, *PD1*, and *Fas*, were inactivated at the genetic level using highly efficient Cas9 adenosine base editor (ABE). Cytotoxicity against tumor lines was first confirmed through *in vitro* serial killing assays, then evaluated *in vivo* using NSG mice challenged with Burkitt's Lymphoma. **Results:** Antibody-stimulated γδ T cell populations yielded >10,000 fold expansion by day 22, and maintained greater TCR diversity than populations derived from zoledronate-based expansion methods (Fig. 1B). γδ T cells were also amenable to highly efficient ABE-mediated triple gene knockout (>90%) without negatively impacting concurrent transposon-mediated CAR integration (>45%). CAR-γδ T cells exhibited potent *in vitro* anti-cancer activity in serial killing assays, and significantly extended mouse survival relative to PBS controls *in vivo* (22±1 vs 47±11 days; $p < 0.0001$; Fig. 1C). Peripheral Vδ1+ γδ T cells were enriched over time in treated mice (Fig. 1D), and exhibited increased persistence when engineered to secrete IL15 (Fig. 1E). Vδ1+ γδ T cell persistence also significantly correlated with survival in IL15-expressing groups (Fig. 1F). Strikingly, a distinct population of αβ TCR-Vδ1-Vδ2- cells representing <7% of injected γδ T cells predominated at endpoint in non-IL15-expressing groups (Fig. 1G). **Conclusions:** High cytolytic activity, potent effector function, and lack of alloreactivity make γδ T cells a prime candidate for use as an off-the-shelf cancer therapeutic. We developed a scalable, clinically-relevant method to expand non-virally engineered human γδ T cells with enhanced cytolytic activity. γδ T cell populations generated using this method achieved >10,000-fold expansion across a 22 day manufacturing process, and, when engineered with a CD19 CAR, displayed highly-efficient killing against tumor lines *in vitro* and *in vivo*. Our polyclonal approach supports improved characterization of discrete γδ T cell populations *in vivo*, informing the development of targeted immunotherapies against diverse subsets of cancer. Ongoing experiments are being conducted to screen for γδ TCRs with the highest reactivity *in vivo*, and to investigate the αβ TCR-Vδ1-Vδ2- cells identified at endpoint.

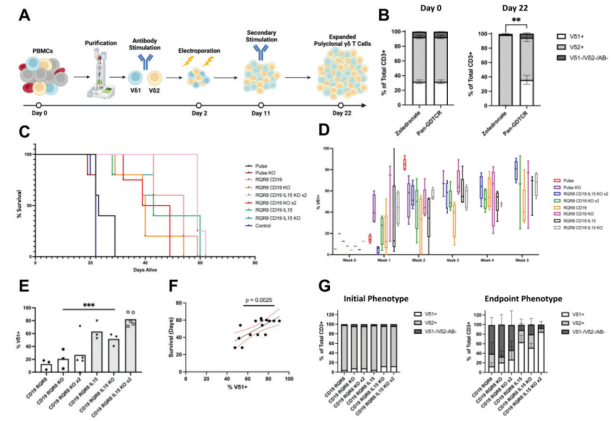

Figure 1. Discrete CAR-γδ T Cell Subsets Mediate Distinct Anti-Tumor Responses *In Vivo*. (**A**) Procedure for large-scale expansion of engineered γδ T cells. Purified γδ T cells were stimulated with pan-γδ TCR antibody and soluble anti-CD28, engineered after 48 hours, re-stimulated on day 11, and harvested at day 22. (**B**) γδ T cells stimulated with pan-γδ TCR possess higher TCR diversity than those produced from zoledronate. (**C**) Survival curve for NSG mice seeded with 10^5 Raji-Luc cells on day -4, then injected with 5 x 10^6 CAR-γδ T cells on day 0. N = 5 mice per condition. (**D**) Ratio of Vδ1+ to Vδ2+ cells through the first five five weeks of treatment. (**E**) CAR constructs containing IL15 yield greater Vd1 outgrowth at endpoint. IL15 production was confirmed via ELISA. (**F**) Endpoint Vd1 expression correlates with improved survival in mice receiving IL15-secreting CAR-γδ T cells. (**G**) Vδ1/Vδ2 expression of T cells at end point. Vδ1-Vδ2- cells also stained negative for αβ TCR. *P* ** > 0.01%, *** > 0.001.

268 Combining Anti-CD33 Chimeric Antigen-Receptor T Cells with the Hypomethylating Agent Decitabine to Treat Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is the most common acute leukemia in adults, causing >10,000 deaths per year in the United States. Current therapies for AML, including hypomethylating agents such as decitabine (DAC), confer an overall long-term survival rate of only 35%, which decreases with patient age. Thus, novel and more effective therapies for patients with relapsed or refractory (r/r) AML remain an urgent unmet need. Targeted, immune-based strategies, including bispecific antibodies and chimeric antigen receptor (CAR) T cells, represent a promising new modality for the treatment of AML. CD33 is a cell surface glycoprotein restricted to early myeloid progenitors and expressed on more than 85% of AML cells, making CD33 an attractive immunotherapeutic target. Several ongoing clinical trials testing CD33-targeted bispecific antibodies and CAR T cells have validated CD33 as a target in AML and have shown favorable safety profiles. However, the clinical outcomes of these trials can be improved upon. Here we present a new CD33-targeted CAR (CD33 CAR) design and our preclinical combination of CD33 CAR T cells with DAC for the treatment of AML. Our platform to generate CD33 CAR T cells uses human CD4/CD8-selected T cells manufactured to express a CD33-CAR with a 4-1BB costimulatory domain. As a single agent, our CD33 CAR T cell induced potent antileukemia responses in vitro against primary AML blasts and tumor cell lines, as well as extended mouse survival compared to mock in our MOLM-14 NSG mouse model of AML. Importantly, our CD33 CAR T cells did not impair normal

hematopoiesis upon co-culture with hematopoietic stem/progenitor cells (HSPCs) cells in a colony forming-unit assay (n=3 HSPC donors), suggesting that our CD33 CAR T cells are unlikely to cause severe hematopoietic toxicity upon infusion in patients. When we tested the combination of CD33 CAR T cells with DAC, we observed enhanced antileukemia activity in vitro when we pretreated tumor cells with 200nM DAC for 3 days prior to coculture with CD33 CAR T cells. Similarly, we found that the in vivo combination of DAC (administered every 24 hrs for 5 days pre CAR T injection) and CD33 CAR T cells extended the median survival of mice to >120 days versus 70 days in mice receiving CD33 CAR T cells alone. To investigate the mechanism underlying the enhanced activity of CD33 CAR T cells following DAC pretreatment, we performed RNAseq analysis on the AML tumor line KG-1a with and without DAC treatment. Although we are currently identifying potential mechanisms, we have detected decreased mRNA transcript levels of DNA methylation machinery (DNMT1 and DNMT3B) and increased DNMT3A upon DAC treatment. Moreover, DAC treatment resulted in downregulation of DNA repair pathways, β-Catenin, and Notch signaling pathways.Taken together, we found that the antileukemia activity of CD33 CAR T cells was enhanced by pretreatment of leukemia cells with DAC. This combination represents a clinically relevant therapy for the treatment of patients with r/r AML.

269 Removal of Endogenous T Cell Receptor (TCR) May Inadvertently Compromise Allogeneic CAR T Cell Function

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Autologous chimeric antigen receptor (CAR) T cell therapy has been a paradigm shift in treating B cell malignancies. Despite its tremendous success in the past decade, the current individualized CAR T manufacture process is complex and has made broader patient accessibility challenging. Allogeneic CAR T cells have attracted huge investments from biotech startups and pharmaceutical industries for their potential as "off-the-shelf" products but until now, these cells have only showed limited persistence comparing to autologous CAR T cells in clinical trials. Generation of allogeneic CAR T cells usually involves ablating the endogenous T Cell Receptor (TCR), via gene editing, to prevent Graft-versus-Host Disease (GvHD). However, whether an intact TCR contributes to optimal CAR functions and persistence has not been thoroughly investigated. In this study, we disrupted the endogenous TCR by two approaches: 1, inserting the CAR-encoding sequence in the TRAC locus via CRISPR-Cas9mediated homologous recombination, using adeno-associated viruses (AAV) as donor templates; 2, directly knocking out TRAC via CRISPR-Cas9 in CAR T cells generated using lentivirus. We demonstrated that inserting the CAR in the TRAC locus under the control of the endogenous TRAC promoter led to reduced antigen-dependent proliferation *in vitro* and decreased tumor killing capacity or persistence in xenograft tumor models, likely due to impaired IL-2 production. This defect was observed with both the 2nd generation CAR tested, anti-CD19 and anti-BCMA CARs, comprising 4-1BB as the co-stimulatory domain. Providing exogenous IL-2 in culture media fully restored the antigen-induced proliferation capacity of the TCR-ablated CAR T cells to the level of TCR intact CAR T cells, generated by lentiviral transduction. We explored strategies to overcome this defect, including inserting CAR at different sites within the TRAC locus, changing the orientation of the CAR expression cassette relative to TRAC transcription, employing either the endogenous TRAC promoter or an exogenous EF1-α promoter to drive CAR expression, replacing 4-1BB with CD28 co-stimulatory domain, and deleting two ITAM domains in the CD3 zeta chain in the CAR construct. Taken together, our data substantiates an earlier report that endogenous TCR promotes *in vivo* persistence of CD19-CAR-T cells (Dana Stenger et.al., DOI: 10.1182/blood.2020005185). We propose that further characterization of the crosstalk between TCR and CAR will guide the design of the next generation of allogeneic CAR T cells, that are as robust as the clinically approved autologous products.

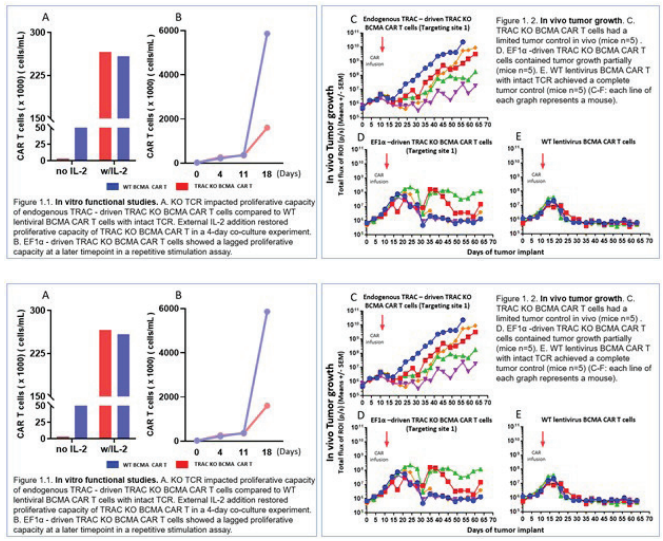

Figure 1.1. **In vitro functional studies.** A. KO TCR impacted proliferative capacity of endogenous TRAC - driven TRAC KO BCMA CAR T cells compared to WT lentiviral BCMA CAR T cells with intact TCR. External IL-2 addition restored proliferative capacity of TRAC KO BCMA CAR T in a 4-day co-culture experiment. B. EF1α - driven TRAC KO BCMA CAR T cells showed a lagged proliferative capacity at a later timepoint in a repetitive stimulation assay.

Figure 1. 2. **In vivo tumor growth**. C. TRAC KO BCMA CAR T cells had a limited tumor control in vivo (mice n=5) . D. EF1α -driven TRAC KO BCMA CAR T cells contained tumor growth partially (mice n=5). E. WT lentivirus BCMA CAR T with intact TCR achieved a complete tumor control (mice n=5) (C-F: each line of each graph represents a mouse).

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AAV Vectors - Preclinical and Proof-of-Concept In vivo Studies II

270 Adeno-Associated Virus Serotype 5 is a Suitable Vector for S100A1-Based Gene Therapy of Post-Ischemic Chronic Cardiac Dysfunction

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Introduction: Toxicity by recombinant adeno-associated viruses (rAAV) in clinical gene therapy trials (e.g., rAAV9-mediated fatal liver failure) can significantly impair the translatability of preclinical rAAV-based cardiac gene therapy programs employing such vectors. In contrast, rAAV5, which continues to demonstrate a beneficial long-term clinical safety profile and scalable producibility has never been systematically evaluated for cardiac-targeted gene therapy.

Hypothesis: We therefore hypothesized that rAAV5 may be a suitable vector for cardiac gene delivery and therapy, respectively, in a large animal heart failure (HF) model that closely approximates human size and cardiovascular pathophysiology. **Approach:** Using rAAV5 and a cardiomyocyte-biased promoter both with a luciferase (luc) reporter for a comparative biodistribution study against rAAV6 and rAAV9 and the human S100A1 gene - as previously validated therapeutic transgene for cardiac gene therapy in various HF models - for a subsequent intervention study, respectively, we determined the suitability of rAAV5 by catheter-based cardiac-targeted administration for sufficient myocardial gene delivery and therapeutic efficacy in normal and post-myocardial infarction (post-MI) HF pigs. **Results:** In a comparative study of rAAV5-luc, rAAV6- and rAAV9-luc in healthy farm pigs (n=5 each group; $1x10^{13}$ vgc per vector per pig), AAV5 achieved a homogeneous cardiac apical-basal transduction pattern that was significantly higher than rAAV9 while rAAV6 resulted in inhomogeneous cardiac transduction, as assessed by luc expression. Relative off-target transduction was significantly higher for rAAV6 and rAAV9 than rAAV5. In a subsequent clinically relevant study that used serial cardiac magnetic resonance imaging (cMRI), we the favorable cardiac transduction pattern of rAAV5 translated in a significant improvement of left ventricular ejection fraction (LVEF) by +19 ± 5 % three months after catheter-based retrograde AAV5-S100A1 gene delivery compared to AAV5-luc in infarcted pigs (n=4 each; $1x10^{13}$ vgc/pig). Moreover, S100A1-treated pigs showed significantly less infarct extension by late gadolinium enhancement (LGE) than controls (-0.5 ± 0.3 g S100A1 vs. +5 ± 1.3 g luc) measured by cardiac MRI. There were no unfavorable alterations in blood counts, clinical chemistry or ECG and S100A1 expression was predominantly contained to the heart. A weighted gene network correlation analysis of myocardial transcriptome data sets from treated pigs unveiled a significant correlation between the improved LVEF and a suppression of inflammatory and immunological pathways ($r=0.96$, $p < 0.01$) and between the absent infarct extension (LGE) and enhanced activity of cardioprotective signaling ($r=-0.82$, $p < 0.05$) by rAAV-S100A1. With injections of 2×10^{11} vgc of AAV5-S100A1 or AAV5-gfp (n=4 each) into the remote myocardium of a post-MI mouse model, we confirmed a significant improvement in echocardiographic LV fractional shortening (+43.8 ± 8.8 %, vs. gfp) and strong suppression of inflammatory gene expression including, i.e., IL1b or TNFa as a novel effect for S100A1 by RT-PCR. **Conclusion:** We conclude that AAV5 is a suitable vector for cardiac-targeted gene therapy and in combination with S100A1 proves effective in treating post-MI cardiac dysfunction in a human sized model. Given the clinical safety profile of rAAV5 and the rather low rAAV5-S100A1 dosage to achieve a lasting therapeutic effect with a clinically applicable route of administration, this vector-transgene combination may be a promising ensemble for a clinical HF gene therapy medicinal product program.

271 Expression and Function of Guanidinoacetate Methyltransferase (GAMT) is Effectively Restored in Cellular and Murine Models of GAMT Creatine Deficiency Following Treatment with *scAAV9.hGAMT*

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Guanidinoacetate methyltransferase (GAMT) is an essential enzyme in the biosynthesis of creatine, a small molecule that plays an important role in energy metabolism. Creatine deficiency disorders are inborn errors of metabolism resulting in several neurological manifestations including developmental delay, intellectual disability, and epilepsy, emphasizing the importance of creatine's role in the brain. Creatine is obtained exogenously and synthesized through the enzymatic function of arginine:glycine amidinotransferase (AGAT) to form guanidinoacetate (GAA) which is then converted to creatine by GAMT. GAMT-deficiency (GAMT-D) is an autosomal recessive disorder resulting in low creatine levels and accumulation of the neurotoxic intermediate, GAA. Current treatment for GAMT-D focuses on restoring creatine levels through oral creatine supplementation and on reducing GAA accumulation through ornithine supplementation to inhibit AGAT function. However, this does not restore creatine or GAA levels in the brain, leaving many of the symptoms unattended. We present a proof-of concept study testing the first central nervous system (CNS)-directed, AAV9-based gene therapy for the treatment of GAMT-D. *In* vitro studies showed that protein and mRNA expression of GAMT were restored resulting in increased intracellular creatine content and reduced GAA accumulation in cellular models of GAMT-D following transfection with the codon-optimized GAMT construct. In a murine model of GAMT-D, treatment with *scAAV9.hGAMT* delivered intrathecally, resulted in increased creatine content as well as significant decreases in GAA accumulation throughout the entire body, including the CNS (Figure 1). Serum collections taken at multiple time-points prior to the short-term endpoint of 13 weeks, showed a marked increase in creatine over time as well as a decrease in GAA accumulation compared to untreated knockouts. GAMT protein expression was restored in the livers of treated animals. This study represents proof-of-principle results for an effective GAA reduction and creatine increase in the CNS using an AAV9 based gene therapy. This therapeutic is currently under further investigation in mouse models to explore its impacts on behaviour and to determine an appropriate therapeutic window for efficacy and safety to allow for translation into human patients.

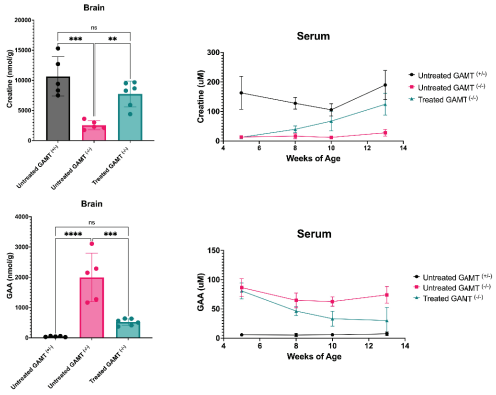

Figure 1. Creatine and GAA levels are corrected following treatment of GAMT knockout mice with *scAAV9.hGAMT* in the brain and serum. Mice were treated with $2.5e^{11}$vg/mouse of *scAAV9.hGAMT* at 6 weeks of age and sacrificed at 13 weeks of age. Proteins extracted from the tissue were analyzed by liquid chromatography tandem mass spectrometry for the presence of creatine and GAA. Significant increases in creatine and decreases in GAA were observed in the brain compared to untreated mice (** $p < 0.006$, *** $p < 0.0002$). Serum was collected at a baseline of 5 weeks of age, every two weeks following injections and at endpoint. Increases in creatine and decreases in GAA were observed over time with significance observed at 10 weeks and endpoint compared to untreated knockouts ($p < 0.002$).

272 Comparative Transcriptomic Analysis Identifies Rescue of Dysregulated Signatures by ATXN1 Knockdown and ATXN1L Overexpression in a Mouse Model of SCA1

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Background: Spinocerebellar Ataxia Type 1 (SCA1) is an adult-onset neurodegenerative disease caused by a (CAG) expansion in the coding region of the ATXN1 gene. The resulting polyglutamine-expanded protein has both toxic gain of function properties, as well as loss of normal ATXN1 activity. We recently demonstrated reversal of disease phenotypes in SCA1 transgenic mice mouse model by overexpression of an ATXN1 paralog, ATXN1-like (ATXN1L), alone or in combination with miRNA-mediated knockdown of hATXN1, delivered by recombinant adeno-associated virus (rAAV) directly to the deep cerebellar nuclei (DCN). In the initial study, two dual expression transgenes were tested, differing in their use of RNA pol II or III driven expression of a hATXN1-targeting microRNA (miS1) and AAV serotype. Despite similar behavioral rescue, we found discordant effects on disease-specific transcriptional signatures between two different dual therapy transgenes and hypothesized that this reflected a difference in packaging capsid and resultant tissue tropism.**Methods:** Here we performed a direct comparison between the two dual SCA1 therapeutic strategies, that combined ATXN1L and simultaneous hATXN1 knockdown by either a mature miS1 arising from RNA pol III transcripts or an RNA pol II transcript intron. Transgenes were packaged into rAAV2/1 and delivered by direct injection into the DCN of B05 mice following onset of motor symptoms. Rescue of motor coordination and gene dysregulation were assessed by behavioral assays and whole transcriptome sequencing of the cerebellum. **Results:** While all strategies led to significant improvements in motor function, expression of human ATXN1L (hATXN1L) alone was not sufficient to normalize dysregulated gene expression signatures in symptomatic B05 mice. This agreed with our previous study, where we also determined that ATXN1 knockdown was necessary for gene normalization. When ATXN1L overexpression was combined with miS1, motor improvements were complemented by robust normalization of disease allele-induced changes in gene expression, regardless of RNA pol II or III usage. Further comparative analysis revealed subtle but significant glial activation and immune gene signatures in the cerebella of mice treated with vectors expressing miS1 under a pol III promoter. No such gene signatures were identified in mice expressing miS1 as an RNA pol II transcript intron. **Conclusion:** In summary, transcriptomic analysis of gene rescue by two dual ATXN1 knockdown strategies suggests that previous contrasting results were due to differing tropism of the AAV capsids. Our data continues to support the combination of both gene therapy strategies into a single rAAV for efficient delivery of a vector capable of overcoming both loss- and gain-of-function mechanisms of disease in SCA1. In addition, we demonstrate the utility of next-generation sequencing to confirm treatment efficacy and inform the development of therapeutic constructs.

273 Optimizing Base Editing Strategies in a Mouse Model of Zellweger Syndrome

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Zellweger Syndrome is a rare disease associated with impaired peroxisome biogenesis and dysfunction caused by mutations in one of 13 genes responsible for peroxisome assembly. Peroxisomes play an important role in a number of cellular functions, including fatty acid metabolism, the catabolism of amino acids, and lipid biosynthesis. The spectrum of Zellweger disorder ranges from mild to severe, affecting a range of organ and tissue function. Patients typically present in early infancy with an incidence of 1 in 50,000 live births in the United States. Symptoms typically includes hypotonia, seizures, vision and hearing loss, as well as life threatening deficits in major organs. Mutations in the gene *PEX1* account for the majority of Zellweger patients and the G843D mutation is one of the most common pathogenic variants. Mouse models have been engineered for a number of Zellweger causative genetic mutations including loss of function alleles and the murine equivalent of the G843D allele in the Pex1 gene. This mouse model displays many of the key features of Zellweger syndrome, including the accumulation of long chain fatty acids. Precision gene editing methods such as base editing and prime editing are promising technologies for the treatment of genetic diseases. Establishing methods for the *in vivo* delivery of new adenine base editors (ABEs) in animal models of human disease is a critical first step before assessing its therapeutic potential. The Liu group has developed therapeutically relevant delivery strategies for base editors *in vivo*, including single-AAV and dual-AAV approaches. Our preliminary data using adenine base editor ABE7.10max (ABEmax) suggest that the dual AAV system can correct *Pex1* G844D mutation *in vivo*. To obtain higher correction levels, we applied recently evolved ABE8e

and ABE8e-V106W, which are more active than ABEmax. These high-activity base editors resulted in a high degree of genome correction with few detected off-target affects. Preclinical efficacy testing in our mouse model resulted in a significant reduction in the levels of very long-chain fatty acid accumulation as well as lipid levels in the liver. These data demonstrate a promising approach for therapeutic base editing as a potential treatment for Zellweger Syndrome.

274 Therapeutic Effect of Linker Protein-Mediated Gene Therapy in a Mouse Model for LAMA2-Related Muscular Dystrophy

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LAMA2-related muscular dystrophy (LAMA2 MD or MDC1A) is the most frequent form of congenital muscular dystrophies. It is caused by mutations in *LAMA2*, the gene encoding laminin-α2, one chain of the heterotrimeric extracellular matrix protein laminin-211 (α2β1γ1). Most patients lack laminin-α2 due to bi-allelic loss-of-function mutations in *LAMA2*. The large size of the coding sequence for laminin-α2 (~ 9.4kb) and the heterotrimeric structure of laminin-211 present a challenge for AAV-mediated gene replacement or gene editing strategies. Here, we describe the development of an AAV-based gene therapy to functionally replace laminin-211 by two small linker proteins. Prior work in transgenic mice has demonstrated that this Simultaneous Expression of Artificial Linkers (SEAL) in skeletal muscle of LAMA2 MD mice has a tremendous ameliorative effect on disease progression (Reinhard et al., 2017. *Sci Transl Med*. 9). As both linkers, mini-agrin (mag) and αLNNd, are small enough to be efficiently packed into AAV, we intravenously co-injected mag- and αLNNd-expressing AAV9 or AAVMYO vectors into LAMA2 MD mice. Both linkers were highly expressed in skeletal muscle and led to a strong improvement of disease phenotypes, including an increase in body- and muscle weights, grip strength, myofiber size and the reduction of fibrosis. Using AAVMYO as a capsid, the same improvement was seen at a ten-fold lower dose than with AAV9. These studies thus establish that systemic delivery of AAVs expressing the two linkers (SEAL technology) might be a possible strategy to treat LAMA2 MD patients. As the linkers are designed from proteins (agrin, nidogen-1 and laminin-α1) that are all expressed in LAMA2 MD patients, this treatment is expected to be well tolerated. Moreover, the fact that the linkers are secreted and act in the extracellular matrix where they also accumulate, suggests that this treatment might be efficacious over a wide dose range of AAV.

275 AAV-Mediated Monoclonal Antibody Expression for the Prevention and Treatment of *Pseudomonas aeruginosa* Infections

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Vector-mediated monoclonal antibody (mAb) expression utilizing adeno-associated virus (AAV) has been shown to provide robust mAb expression from a single intramuscular (IM) dose. Our novel triple mutant capsid, AAV6.2FF, has been shown to provide long-term therapeutic mAb expression against infectious pathogens in numerous animal models. AAV6.2FF is capable of effectively transducing the lungs, therefore we aimed to target *Pseudomonas aeruginosa* - a nosocomial pathogen of global concern, that is particularly prevalent in patients suffering from cystic fibrosis (CF). Roughly 40% of patients with CF have chronic *P. aeruginosa* infections when reaching adulthood, which is associated with worsening lung function and increased mortality. Antibiotic treatment is the first line of defense when *P. aeruginosa* is first detected, although successful eradication is variable and not sustained due to the intrinsic and acquired resistance of *P. aeruginosa* towards multiple classes of antibiotics. To date, there are no licensed vaccines for this pathogen, prompting the urgent need for novel treatment approaches to interfere with *P. aeruginosa* infection and persistence. A highly protective mAb targeting exopolysaccharide Psl has been shown to mediate serotype-independent opsonophagocytic killing of *P. aeruginosa.* Psl is abundantly expressed in both mucoid and non-mucoid isolates, and is suggested to be involved in host attachment, immune evasion and biofilm formation. The PcrV protein located at the apex of the type-III secretion system injectosome is integral for the translocation of effector molecules into the host cell cytoplasm, and has also been shown to be a highly potent mAb target. Given the critical role of Psl and PcrV in the establishment of *P. aeruginosa* infections, it was rationalized that a combination of both anti-Psl and anti-PcrV mAbs engineered into a single bispecific mAb would enhance strain coverage and reduce bacterial burden. Bispecific hIgG antibody (MEDI3902) which combines single-chain variable fragments of both anti-Psl and anti-PcrV mAbs has been found to provide protection in prevention and treatment of *P. aeruginosa* infections *in vivo* and was evaluated in a Phase II clinical trial (NCT02696902). Here we have validated the AAV6.2FF platform as a strategy to generate long-term antibody expression beyond 12 weeks post-vector administration. Our three vectors of interest, AAV6.2FF-PcrV, AAV6.2FF-Psl and AAV6.2FF-MEDI3902 provided significant protection in a lethal challenge model against *P. aeruginosa* strains PA01 and PA14. BALB/c mice IM injected with $1x10^{11}$ vector genomes (vg) of AAV6.2FF-PcrV provided 100% protection from a lethal intranasal challenge of $4.47x10^{7}$CFU PA01. AAV6.2FF-mediated expression of these antibodies dramatically reduced bacterial burden *P. aeruginosa* dissemination to other organs in a lethal PA01 challenge model. All vectors provided significant protection, although notably the bispecific vector provided significantly greater protection against bacterial dissemination to the liver, lung, spleen, and blood compared to the monospecific vectors. Together, these results support further investigation of utilizing AAV-mediated

mAb expression for the prevention and treatment of *P. aeruginosa* infections and other bacterial pathogens of public health concern, in which current treatment strategies are limited.

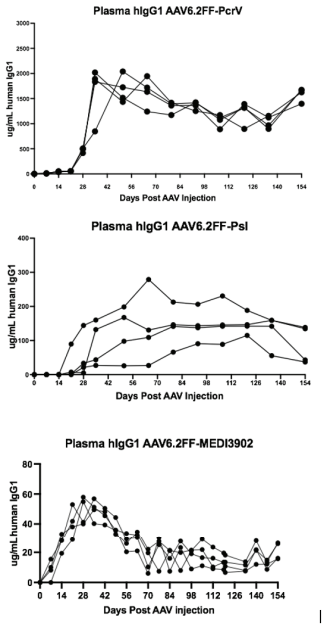

Figure 1: **Serum antibody expression levels after intramuscular administration of 1x10^{11} vg of AAV6.2FF-PcrV, AAV6.2FF-PsI or AAV6.2FF-MEDI3902.** BALB/c mice (n=4) were IM administered 1x10^{11} vg of AAV6.2FF-PcrV (A), AAV6.2FF-PsI (B), or AAV6.2FF-MEDI3902 (C), and their serum human IgG levels were monitored for 22 weeks.

276 AAV9-Delivered Artificial miRNA Gene Therapy Rescues the PS19 Mouse Model of Tauopathy

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Tau protein is expressed by the microtubule-associated protein tau (*MAPT*) gene and plays an essential role in the central nervous system. Aging, environmental stress, and *MAPT* mutations can alter Tau expression and its post-translational modifications, leading to abnormal accumulation of tau proteins and CNS dysfunctions (characterized as tauopathies). Tauopathies are presented in a spectrum of neurodegenerative diseases (e.g. Alzheimer's disease and frontotemporal dementia), and have been associated with cognitive decline in both clinical studies and animal models. Based on WHO surveys, more than 55.2 million people worldwide suffer from dementia, with a predicted increase to 139 million in 2050, suggesting an urgent need for treating tauopathies. To treat tauopathies, we propose a tau-reduction therapy, which is the use of adeno-associated viral (AAV)-mediated gene silencing therapy. To reduce tau levels, we developed panels of artificial, tau-specific microRNA (miRNA) shuttles that were designed to target mRNA for all isoforms of tau found in the human or mouse brain. We previously selected lead candidates based on tau reduction at both mRNA and protein levels and packaged them into AAV9 vectors. The therapeutic effects of AAV9-hTaui were tested in a commonly used mouse model of tauopathy, PS19 mice, which carry the 1N4R isoform of human MAPT with the P301S disease-associated mutation. PS19 mice express human MAPT at a 7 times higher level than the endogenous mouse Mapt, and they exhibit significant tau pathology, neurodegeneration, loss of body weight and progressive hind-limb paralysis around 9-12M of age. We tested efficacy of our treatment by delivering AAV9-hTaui to PS19 mice at 3M, 6M and 9M of age. Treatment groups included WT littermates treated with vehicle or AAV-Scr and PS19 mice treated with vehicle, AAV-Scr or AAV9-hTaui. Survival rate, functional tests, and BW changes were evaluated 3M after the intra-cisterna magna injection. Tau mRNA and protein levels in brain tissues were assayed using RT-qPCR and ELISA. Additionally, a cell-based tau seeding assay was used to measure the presence of pathological tau in brain lysates. Tau pathology and neurodegeneration in the brains of mice treated at 9M of age were also assessed by histology. We found that the tau mRNA and protein levels were significantly reduced in the AAV9-hTaui group and were unaffected in the AAV-Scr groups. Consistently, the tau seeding assay showed reduced levels of pathological tau in brains from PS19 mice treated with AAV9-hTaui and was unchanged in the control groups. At functional output levels, our one-time treatment was able to rescue the survival rate, loss in body weight, and the hind limb clasping phenotype in the PS19 mice. In a separate safety study, AAV9 carrying a mouse-specific Tau miRNA (mTaui) was ICM delivered to 3M old WT mice to determine how knock-down of endogenous tau is tolerated in the adult brain. In life assessments supported that tau reduction did not affect weight gain, survival, hind-limb clasping, or behaviors related to motor function, anxiety and cognition up to 12 months post-dosing. Histopathology of tissues are ongoing. Together, this study presents a one-time, vectorized delivery of a tau-specific miRNA as a potential treatment for tauopathies.

Vector Product Engineering, Development, and Manufacturing

277 A Sequence-Upgraded PolyA LTR (SupA-LTR™) Imparts Enhanced Transcriptional Insulation to New 4th Generation Lentiviral Vectors from Both 5' and 3' Genic Regions in Target Cells

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The structure of 3rd generation lentiviral vectors (LVVs) has not appreciably changed over the last two decades, and such LVVs have been widely adopted for *ex vivo* applications. The development of LVVs for use in directly administered *in vivo* applications is being re-invigorated through more specific targeting and improvements in both production scale and product quality. Indeed, regulatory authorities are placing very significant importance upon on the safety and quality aspects of viral vector gene therapy products generally. Consequently, we have been seeking to improve LVV genome design to further mitigate potential risks associated with high dose applications.

An unintended consequence of the deletion of U3 promoter sequences within SIN-LTRs was the removal of overlapping polyadenylation enhancer sequences, leading to reduced transcription termination efficiency with SIN-LTRs compared to the full length LTR. Termination at the native polyA signal within the R region is not sufficiently rescued by retention of the native 'GU-rich' downstream enhancer (DSE) within U5. This results in increased transcriptional read-in/out of the integrated LVV cassette, and concomitant interactions with the host transcriptome, as well as some potential effects on transgene expression. Moreover, there is currently no mechanism to control transcription read-in on the anti-sense strand, and therefore some integration events may be lost to dsRNA formation and associated innate responses. Others have improved polyadenylation efficiency on the sense strand by insertion of heterologous upstream enhancer elements (USE) within the SIN region. Here we have developed 'sequence-upgraded polyA-LTRs' (supA-LTRs) that not only contain a USE but also an optimally placed DSE on the sense strand. This is achieved by two modifications within the LVV expression cassette: [1] the careful insertion of a synthetic DSE into the 5' R region, and [2] the use of an 'R-embedded' heterologous polyadenylation sequence to replace the 3' R-U5 sequence entirely, thereby placing the polyA signal upstream of R sequences required for 1st strand transfer. Due to the spatial nuances of polyA-cleavage of vector genomic RNA, and subsequent reverse-transcription, the resultant supA-LTRs that flank the integrated cassette harbour an optimally spaced USE-pA-DSE configuration. Additionally, an inverted polyA-DSE is incorporated, so that the integrated LVV cassette is also transcriptionally insulated from the 3' direction. This results in up to 50-fold and 100-fold reduction of transcription through supA-LTRs compared to SIN-LTRs in sense and anti-sense, respectively. Interestingly, the greatest block to 5' transcription read-in to the integrated LVV cassette is observed when supA-LTRs are combined with other features of our 4th generation LVVs that have an inactivated major splice donor site within the packaging signal. Additionally, we find a modest but beneficial increase in transgene expression of up to 2-fold when employing supA-LTRs. Further aspects to the characterisation of supA-LTRs in LVV production and in target cells will be presented. We anticipate the use of supA-LTRs will be of great assistance to the field in providing additional safety and utility to the next generation of LVVs.

278 Fitting-Out 4th Generation Lentiviral Vectors: New Technology to Improve Their Production, Quality, Safety, Capacity and Utility

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The development of Lentiviral vectors (LVVs) based on HIV-1 has focussed on a 3rd generation vector structure that has not appreciably changed over the last two decades. The clinical success in using LVVs to deliver therapeutic genes to dividing and non-dividing cells is built upon their safety features. Nevertheless, as clinical use expands there is a need to continually improve product quality and safety, and researchers are seeking to produce and deliver LVVs with ever larger, more complex transgene cassettes. Here we provide a summary of a suite of new technologies that can be adopted to create a new class of 4th generation LVV genomes where vector production, quality, safety, capacity and utility are further improved. Our 4th generation LVVs come in two core designs: [1] Rev-dependent or '2KO-LVVs' and [2] Rev-independent or 'MaxPax' LVVs. Both of these genome types harbour the same key modification of an inactivated major splice donor (MSD) site within the packaging sequence. We and others have shown that the MSD within 3rd generation LVVs can aberrantly splice to transgene cassette splice acceptor sequences during LVV production, leading to less full-length vector genomic RNA (vRNA), and a substantial amount of truncated (transgene encoding) vRNA. We show that 2KO-LVVs and MaxPax-LVVs do not produce such truncated RNAs, and consequently contain a far higher proportion of full length vRNA within resulting LVV particles. Since MSD-inactivation alone leads to a reduction in LVV out-put titres, we have engineered different solutions to overcome this in each 4th generation LVV type. 2KO-LVVs titres are maximised by the co-expression of a modified U1 snRNA that can bind to the LVV packaging sequence during production, which effectively serves as a chaperone-like molecule to increase the pool of packageable vRNA for assembly. For MaxPax-LVVs, the attenuating effects of the MSD-inactivation are abrogated by the use of a synthetic intron or 'Vector-Intron' (VI) to replace the Rev-response element. The splicing-out of the VI during pre-vRNA biogenesis stabilises the vRNA and consequently does not contribute to overall vRNA length - an important factor in maintaining RNA pool steady state levels and vRNA packaging efficiency. Thus, together with other deletions within the packaging sequence, MaxPax-LVVs can package ~1kb additional sequence, increasing transgene capacity. Since transgene cassette introns cannot be retained by MaxPax, this vector is ideal for very large or multiple transgene payloads where minimal cis-acting elements are employed, and introns are not required. Where transgene introns are desirable, the 2KO-LVV design should be considered. Both of these 4th generation LVV genome types employ 'sequence-upgraded polyA' or supA LTRs, providing improved transcriptional insulation for the integrated LVV in patient cells. Current 3rd generation LVVs employ self-inactivating (SIN) LTRs that are not only deleted in HIV-1 U3 promoter sequences to improve safety, but concomitantly lack polyA enhancer sequences required for efficient transgene transcription termination. These supA-LTRs have been configured to reduce transcriptional read-in to the cassette from chromatin by up to 50-fold from both 5' and 3' directions, as well as increase transgene expression by up to 2-fold. Layered on top of these other features is the option to employ the 'Transgene Repression In vector Production' system. The TRiP system™ works maximally within these 4th generation LVVs to minimise the variable effects of transgene expression during LVV production (doi: 10.1038/ncomms14834). Employing TRiP can enhance out-put titres, simplify downstream processing, and ensure that transgene protein is not embedded within final LVV product.

279 Suspension-Based Adenovirus Production Process Development and Scale-Up in scale-™ Fixed-Bed Bioreactors

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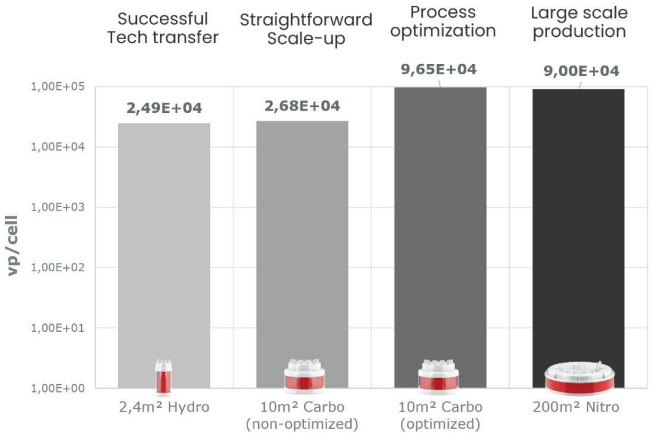
Suspension-cultured HEK293 cells and serum-free conditions have become an industrial standard for adenovirus production. To maximize the virus titers, a good cell state and high cell density are required. Commonly operated in batch, cell density achieved is low and the implementation of a more complex feeding strategy can be reached only with an external perfusion system. In this study, the manufacturing of a non-replicative Gorilla Adenovirus vector (GRAd, proprietary of ReiThera) in suspension cells was adapted to the scale-X™ bioreactor. The scale-X™ fixed-bed bioreactor family has been designed to ensure flexible and scalable manufacturing for high cell density processes without a need for additional feeding systems. The proof of concept showed a direct adaptation of the suspension cell-based process to scale-X™ hydro (2.4m²). The cells were entrapped in the fixed-bed structure with the productivity of 2.49 x 10^4 viral particle (VP)/cell (figure 1). The intermediate scale (scale-X™ carbo 10m²) enabled a linear process scale-up. The process optimization by higher cell density at infection, production phase and medium volume increased the viral particle titers to 9.65 x 10^4 VP/cell. Further scale-up to 200m² scale using scale-X™ nitro bioreactor, never performed for adenovirus production, led to a large-scale consistent cell growth and titer of 9.00 x 10^4 VP/cell (total yield 3.53x10^{16} VPs). As a conclusion, the scale-X™ family provided an efficient way to make process transfer on small scale, proved straightforward scalability and process optimization on the middle scale, and yielded high titer adenovirus manufacturing on a large scale.



280 Lentiviral Vector Production Using Suspension Adapted HEK293T Cells in Serum Free Media

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As more cell and gene therapies make their way to the market, there will be an increasing demand for clinical grade lentiviral vectors. Adherent HEK293T cells have been used in vector manufacture but as batch sizes increase, the required surface area makes the process cumbersome and expensive. To solve this, we generated a suspension adapted HEK293T cell line HEK293T(IUS) able to grow in serum-free conditions and evaluated the line's potential to generate high titer vector. A series of three Design of Experiment (DOE) trials were performed to optimize transfection conditions of this new cell line. The optimized conditions were then used in a WAVE bioreactor to successfully produce three 4-5-liter batches of vector product. Downstream processing with tangential flow filtration generated diafiltered, concentrated material which was evaluated for vector physical and infectious titer. **Methods:** HEK293T(IUS) cells were transiently transfected with a four plasmid, third generation, VSV-G pseudo typed lentivirus vector expressing GFP. PEIpro® transfection reagent and sodium butyrate were used to enhance transfection. JMP software was used for the DOE experiments which compared the effects of several culture parameters on transfection efficiency and vector titer. Physical titer was determined by p24 assay. To determine a functional titer, adherent HEK293 cells were transduced with the vector supernatant and GFP expression was measured by flow cytometry. Following the results of the DOE, the culture volume was increased twenty times from 15mL to 300mL in shake flasks using the optimized conditions. Three 4-5 L lentiviral vector productions were then performed using the WAVE bioreactor platform and the optimized transfection conditions. The cells were expanded in shake flasks and a WAVE bag until enough cells were generated to begin the transfection. The transfection was then performed in the WAVE bag according to the DOE optimized parameters. After the optimized time period, the supernatant was collected, clarified, treated with DNAse, diafiltered, and concentrated using TFF. **Results and Conclusion:** The DOE compared the effects of several transfection parameters on the functional titer. The use of Sigmacoat® did not significantly increase vector titer. The only significant two factor interactions found were the interactions polyplexing time with Pluronic acid concentration leading to a reduction in titer (p=0.02165 and F Ratio=9.48) and the interaction between plasmid DNA concentration per 10^6 cells and the PEI volume per 10^6 cells increasing the titer (p=0.009, F Ratio=12.38). The optimal conditions were found to be 3 million cells per mL, 0.25 µg plasmid DNA per 10^6 cells, 1.0 µL PEIpro® per 10^6 cells, 5 minutes polyplexing, and no media change. The result of the 300mL cultures were found to be slightly better than a 15mL scale indicating larger scale up is possible. The infectious titers of the WAVE bag were compared to an optimized clinical adherent cell production run and were found

to be similar. Further development could better tailor the procedure to the features of the WAVE reactor thus improving yield generated and allowing scalability to higher volumes. In conclusion, we successfully optimized the transient transfection of a suspension adapted HEK293T cell line. We also demonstrated the production feasibility of 5L in a WAVE bioreactor. The HEK293T(IUS) cell line will be made available from the NGVB (www.NGVBCC.org).

281 Development of a Scalable Lentiviral Manufacturing Upstream Process with a Suspension Producer Line for Cell Therapy Applications

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In the last years, lentiviral vectors have advanced from being a genome delivery tool to becoming a vital part of several cell therapies. With the fast evolution of the cell- therapy field, new demands have emerged, which cannot be met using conventional lentiviral manufacturing technologies. To enable the development of affordable and effective cell therapies, well known lentiviral manufacturing bottlenecks, e.g., predictable upscaling processes, maximized recovery rates and high functional titers, need to be addressed. Here, we focus on upstream and downstream scalability. A key component of a scalable upstream process is a suspension producer cell line. We analyzed the productivity of two commercially available suspension cell clones, ThermoFisher's Virus Producing Cells (VPC) 2.0 and 1.0 (data not shown), as well as an adherent cell clone (HEK-T from CLS) used as control. The productivity studies of VPC 2.0 were conducted using the commercially available reagents, i.e., Viral Production medium and the LV-MAX™ Transfection Kit. The reagents for culturing and transfecting the adherent cell line were DMEM supplemented with 2 mM glutamine and PEI. Per VPC 2.0 cell, the number of lentiviral particles (i.e., genome copies or GC measured by qPCR) found in the crude fraction was in average 4,1E3 (n=3; culture volume = 240 mL; total number of cells on day of transfection = 1,1E9). While per adherent cell was 1,6E3 genome copies (n= 3; area= 6,320 cm2; total number of cells on day of transfection = 6,6E8). Hence, the productivity of the VPC 2.0 was 2.5-fold higher than that of the adherent cell line. Next, we tested if the productivity of VPC 2.0 is affected during the upscaling by using the following volumes: 30, 60, 120, and 240 mL. The total number of lentiviral particles in the crude fraction (i.e., productivity) were 2.6E9 GC/mL (n=3) for the 30 mL; 2.7E+09 GC/mL (n=3) for 60 mL; 1.9E+09 GC/mL (n=3) for 120 mL and 2.7E7 GC/mL (n=3) for 240 mL. The productivity of VPC 2.0 cell, therefore, was not affected by upscaling the culture volume, except for the 120 mL condition. Next, we focused on the scalability of the clarification process by analyzing two different methods: ultrafiltration and filtration using the SartoClear Dynamic kits with diatomaceous earth as a filter aid. Although recovery rates were similar for both methods, clarification via diatomaceous earth reduced the processing times, which is a prerequisite for clarifying large volumes. Further work is planned to optimize the scalability of the subsequent downstream steps. This includes testing scalable ion exchange monoliths and an affinity purification method based on an agarose base-bead, e.g., CaptureSelect™ Lenti VSVG Affinity Matrix. This study illustrates that the strategic development of upstream and downstream processes is key to eliminating lentiviral manufacturing bottlenecks, a must for the development of affordable and effective cell therapies.

282 Intensified Production Process for Gorilla Adenoviral Vector GRAd: Yield, Purity, Potency and Stability Results

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Simian adenoviruses derived from the chimpanzee, bonobo, and gorilla, are not known to circulate and/or cause pathological illness in humans, consequently have low/no seroprevalence (0%-18%) in the human population and therefore it can be ideal viral vectors for vaccine and advanced therapy applications. Adenoviral vectors derived from simian adenovirus and more specifically from great apes have been studied in millions of human subjects using vaccine vectors encoding different antigens (relevant to Ebola, malaria, hepatitis C, human immunodeficiency virus [HIV], respiratory syncytial virus [RSV] and COVID-19) have shown that this vaccine platforms are safe and can generate potent, durable, and high-quality T cell and antibody (Ab) responses.Reithera has developed a COVID-19 vaccine using a novel gorilla adenovirus classified into species C. This adenovirus isolated from a captive animal was converted into a replication-defective adenoviral vector generating GRAd-COV2 vaccine. GRAd-COV2 has completed the phase II clinical trial in 2022 showing a good safety profile and a strong immunogenicity.In parallel Reithera has developed a production process based on the proprietary high yield packaging cell line ReiCell 35S based on low passage HEK293 cells. The process was designed to provide high volumetric yield by exploiting perfusion and high cell density at infection in single use stirred tank bioreactor. The downstream platform was designed on orthogonal purification process based on selective host cell DNA precipitation step and cell debris removal by depth filtration followed by anion exchange membrane chromatography, HCDNA trimming step by endonuclease digestion, mixed mode polishing chromatography and formulation by TFF. The process was set up in 2L bioreactor and scaled up to 1000L and 2000L single use STR. The analytical results demonstrated that the process provides a very high yield (>60%) with the final product meeting all specification in terms of potency and purity. In addition, the final product was demonstrated to be stable at +2-8°C for more than one year. In conclusion, this novel GRAd vector is an attractive vaccine platform suitable for development and production of high-throughput vaccines against pathogens or diseases requiring the generation of a well-coordinated immune response.

283 Lentiviral Vector Engineering: Impact on Manufacturing and Vector Performance

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Background and novelty Advanced therapy medicinal products transformed medicine, and past unmet medical needs are now being treated with cell and gene therapy approaches targeting the cause of the disease. Gene therapies are based on the transfer of genetic material to the patients' cells and lentiviral (LV) vectors are one of the preferred delivery systems, particularly when targeting hematopoietic cells (e.g. hematopoietic stem cells, T lymphocytes). LV vector engineering can be used to improve their efficacy and manufacturing. Herein, we describe vector engineering strategies, at the level of LV enzyme and envelope glycoproteins, enabling constitutive manufacturing through stable cell lines and effective T-cell transduction. **Experimental approach** The ideal LV vector manufacturing process should rely on stable producer cell lines and perfusion systems, enabling high cell density and long-term production. In contrast, most bioprocesses for LV vectors are either based on transient transfections or inducible producer cell lines that generally constrain production to short-term runs. LV vector protease and VSV-G envelope glycoprotein are cytotoxic, challenging the development of constitutive stable cell lines. To address these obstacles, we either eliminated or reduced the cytotoxicity of LV vector components by engineering less active LV protease and highly efficient non-toxic envelope glycoproteins. The LV vector protease was engineered by mutating the catalytic center at amino acid 26. As an alternative to the VSV-G envelope, gammaretrovirus glycoproteins (4070A, GaLV, and RD114) were evaluated and engineered to pseudotype LV vectors. These glycoproteins do not induce cytotoxicity, ultimately allowing the development of stable LV producer cells. **Results and discussion** Reducing LV cytotoxicity enabled the use of constitutive promoters. The mutated protease T26S generated similar titers to the wild-type one when using VSV-G, but not when pseudotyping with gammaretrovirus glycoproteins. Novel 4070A, RD114, and GaLV-derived glycoproteins were developed with the aim of improving its cleavage by T26S protease and increasing LV vector titers. Engineering the glycoproteins enable to overcome titer differences observed between LV vector productions using wild-type and T26S protease. Transduction of human peripheral blood mononuclear cells (PBMCs) with pseudotyped LV vectors 4070A, RD114, and GaLV novel envelopes showed successful T-cell modification, and at similar efficiencies as obtained with VSV-G LV vectors. Proof-of-concept LV vector producer cell lines constitutively and stably producing infective titers above 10^6 $TU.mL^{-1}.day^{-1}$ were successfully established. The producer cells showed to be stable over 3 months of subculture. Bioreaction studies showed continuous vector production over 10 days. Process intensification, enabling high cell densities, through perfusion resulted in improvement of titer to 10^7 $TU.mL^{-1}.day^{-1}$. This work shows LV vector engineering can enable the development of more effective manufacturing processes and improve vector performance.

Cancer - Oncolytic Viruses

291 A Personalised Neo-Antigen Viro-Immunotherapy Platform for Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) corresponds to approximately 20% of all breast tumours, with a high propensity for metastasis and a poor prognosis. Because TNBC displays a high mutational load compared to other breast cancer types, a neo-antigen-based immunotherapy strategy could be effective. One major bottleneck in the development of a neo-antigen-based vaccine for TNBC is the selection of the best targets, *i.e.*, tumour-specific neo-antigens which are presented at the surface of tumour cells and capable of eliciting robust immune responses. Here, we used bioinformatic tools and cell-based assays to identify immunogenic neo-antigens that can be presented by the patients' HLA class I alleles. We further validated our approach by generating neo-antigen-specific CD8+ T-cells capable of recognising a human TNBC cell line expressing the mutated gene. A further hurdle to overcome in development of novel neo-antigen-based therapies is delivery, as this process must engage and enhance immune activation against antigens. We have developed a new generation of a highly tumour-specific oncolytic Vaccinia virus (VV) and show that it can directly destroy tumour cells independently of antigen expression, change the tumour microenvironment and attract effector T-cells and maintain their activation. Using a preclinical model of TNBC we show that long peptides encompassing neo-antigens can be delivered in a mixed solution with an oncolytic VV in a prime/boost regimen to induce neo-antigen-specific CD8+ T-cell response, slow tumour growth and increase survival.

292 Emerging Data from the Use of TILT-123, an Oncolytic Adenovirus Armed with TNFa and IL-2 in Patients with Solid Tumors

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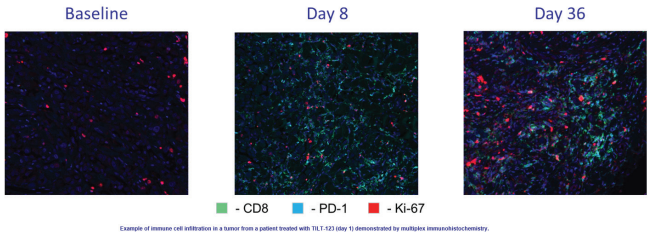
The therapeutic landscape in oncology has notably changed in the last decade after the approvals of immunotherapeutic drugs such as immune checkpoint inhibitors (ICIs) and other T-cell therapies. The

myriad of clinical trials conducted increasingly allow us to understand how and when to use those therapies. One of the major limitations discovered is that many patients do not respond to immunotherapies. TILT-123 (Ad5/3-E2F-d24-hTNFa-IRES-hIL2) is an oncolytic adenovirus engineered to increase the activity of the immune system towards the tumor, and to unlock efficacy for subsequent immunotherapies, in patients that otherwise do not benefit. Over a decade of lead optimization and preclinical testing, paved the way for clinical studies to assess safety and efficacy of TILT-123. Since 2020, four Phase I studies have been initiated: a monotherapy (NCT04695327) and combinations with TILs (NCT04217473), pembrolizumab or avelumab (NCT05271318, NCT05222932). Two of the trials (NCT04695327, NCT04217473) were phase I open-label, dose-escalation clinical trials, that have the primary endpoint of establishing safety of the treatments in patients with injectable advanced refractory and recurrent solid tumors, that cannot be treated with curative intent. The secondary endpoints include correlative analyses performed to better understand the pharmacokinetic and pharmacodynamic properties of TILT-123. Virus specific detection techniques were utilized in different biological samples such as tumor, blood, urine, and saliva at various timepoints following drug administration, in order to understand the presence and persistence of the agent. At the same time, cell phenotyping, proteomic and transcriptomic effects were evaluated on available samples to measure the immunological impact in the tumor milieu. RECIST 1.1, iRECIST, and a PET-based criteria were used to assess efficacy. Interim data (n=18 patients) supports the safety of TILT-123 and no DLTs were reported. Expected adverse events associated with oncolytic adenoviruses such as fever, nausea, chills, and fatigue, appeared typically as low grade according to the CTCAE v5.0. Antitumor efficacy derived from the use of TILT-123 has been observed in several patients. The dose escalation had a direct correlation with the increased detection of the virus in blood up to 14 days post administration. Cytokine and chemokine (IL-12, CXCL9, CXCL10, CXCL11, CXC3CL1, CCL3, CCL4) signatures were observed in blood suggesting that the treatment triggered immune trafficking and activity. Likewise, analysis of tumor samples revealed increasing diversity of CD8 and CD4 T lymphocyte subsets.



Example of immune cell infiltration in a tumor from a patient treated with TILT-123 (day 1) demonstrated by multiplex immunohistochemistry.

Emerging data from clinical trials suggests that TILT-123 has an adequate safety profile and can reshape the tumor microenvironment resulting in antitumor activity, at the tested doses. Studying biological samples allowed a better understanding on the mechanism of action of this gene therapy product.

293 Addition of αTIGIT Checkpoint Blockade to MV-s-NAP-uPA + αPD-1 Combination Immunotherapy Enhances Long-Lasting Immunity in a Syngeneic Murine Glioblastoma Model

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Introduction: Glioblastoma multiforme (GBM) remains the most aggressive and lethal primary brain tumor with dismal survival despite multimodality treatment. GBM is an immunologically "cold" tumor, marked by heterogeneity, few tumor-specific neoantigens, and poor immune cell infiltration. Attempts to treat GBM with immune checkpoint blockade (ICB) therapy against programmed death receptor 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) have been largely unsuccessful. Our group has previously shown that combination of an engineered oncolytic measles virus strain, MV-s-NAP-uPA, and αPD-1 ICB significantly increases survival and immune activation in syngeneic glioma models. Neutrophil activating protein (NAP) derived from *Helicobacter pylori* is an immunostimulatory protein and can elicit strong Th1 immune responses. Inclusion of NAP in the MV backbone enhances its immunostimulatory potential. Analysis of GBM-bearing mice treated with αPD-1 with or without MV-s-NAP-uPA revealed upregulation of the checkpoint T cell immunoglobulin and ITIM domain (TIGIT), but not T cell immunoglobulin and mucin-domain containing 3 (TIM-3) or CTLA-4 on several immune populations, including macrophages, CD4 and CD8 T cells. TIGIT binds to CD155 and CD112; CD155 is documented to be overexpressed in several tumors including GBM. TIGIT-receptor ligation induces immunosuppressed phenotypes in CD4 and CD8 T cells, natural killer (NK) cells, and macrophages. We hypothesized that combined anti-PD1 and anti-TIGIT blockade in combination with MV-s-NAP immunovirotherapy would result in synergistic efficacy**.** **Methods:** The previously described engineered measles strain MV-s-NAP-uPA is capable of infecting murine cells via urokinase-type plasminogen activator receptor (uPAR) and expresses a secretory form of NAP (s-NAP). C57/BL6 female mice were implanted with syngeneic CT-2A murine glioblastoma cells orthotopically and then treated twice with MV-s-NAP-uPA intratumorally. Mice received αPD-1 and/or αTIGIT ICB therapy on a 3-day cycle for 3 or 6 treatments. Mice were followed for survival and rechallenge or were sacrificed at pre-determined timepoints for analysis via immunohistochemistry (IHC), flow cytometry, or cytometry by time of flight (CyTOF). Cytokine analysis was performed on plasma samples from various timepoints. Mice were rechallenged six months later with CT-2A or B16-F10 melanoma intracranially to evaluate tumor-specific long-term immune memory and response. **Results:** MV-s-NAP-uPA, αPD-1, and αTIGIT combinatorial therapy resulted in near 100% survival rates, superior to all other tested regimens. Cytokine analysis revealed a Th1-skewed systemic cytokine milieu upon combination treatment. CyTOF analysis is ongoing; findings will be associated with survival outcomes. Rechallenge experiments with surviving mice showed that the anti-tumor response is CT-2A-specific, suggesting a long-lasting tumor-specific immunity. **Conclusions:** Triple combination therapy with MV-s-NAP-uPA, αPD-1, and αTIGIT appears to be the most

promising treatment evaluated in this study. These mice survive long-term and do not exhibit detectable side effects. Correlative analysis is ongoing. We are also currently assessing any toxicological constraints of this triple therapy prior to clinical translation.

294 A First-in-Human Phase I Clinical Trial of Intratumoral Administration of a Measles Virus Derivative Expressing the *Helicobacter pylori* Neutrophil-Activating Protein in Patients with Metastatic Breast Cancer

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Background: Engineering measles virus (MV) to express secretory *Helicobacter pylori* neutrophil-activating protein (s-NAP) enhances the immunostimulatory potential of MV. We present safety results from a first-in-human phase 1 trial of MV-s-NAP in women with metastatic breast cancer (MBC). **Methods:**A 3+3 Phase I clinical trial was conducted to determine the maximally tolerated dose (MTD) of a single intratumoral administration into a single lesion of MV-s-NAP. Dose-limiting toxicities (DLTs) included: grade 3+ hematologic/non-hematologic toxicity, viremia > 6 weeks from viral administration, and serum creatinine > 2 x baseline, at least probably related to treatment. Participants are observed for 3 months following injection for Viral shedding (tested by qRT-PCR in mouth gargle and urine), viral replication in the tumor (using Vero cell overlay assay), high mobility group box 1protein(HMGB1) expression and immune response to MV and NAP transgene (by ELISA and virus neutralization assay) and then for 2 years for disease events. **Results:**Seven women with ER+/Her2+ MBC, were enrolled as of January 30, 2023; three at dose level 1 (DL1: $TCID_{50}$:$1x10^8$), three at DL2 ($TCID_{50}$:$3x10^8$), and one at DL3 ($TCID_{50}$:$1x10^9$). Data are available for DL1-2. No dose-limiting toxicities (DLT) were observed. The most common grade 1-2 toxicities reported were: tumor pain (5 pts), and nausea (2 pts). No evidence of shedding was seen. Viral replication was detected in two patients on day 3, including one being a 3-fold increase of serum HMGB1 on day 8 persisting 6 weeks. There was no detectable increase in anti-NAP antibodies. All 6 of these patients have progressed. **Conclusions:** No DLTs were observed for DL1-2 of MV-NAP. Moreover, no detectable increase in anti-NAP was seen with viral replication seen in 2 of 6 patients.

295 Temozolomide Chemotherapy Enhances Dual Blockade of an Oncolytic Virus Expressing an Anti-CD47 Antibody for Breast Cancer Brain Metastases

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Limited therapeutic options are available for breast cancer brain metastases (BCBM). Thus, there is an urgent need for novel approaches. We previously engineered an effective oncolytic herpes simplex virus-1 (oHSV) expressing a full-length anti-CD47 monoclonal antibody (mAb) with a human IgG1 scaffold (OV-αCD47-G1) to treat ovarian cancer and glioblastoma (*Nat Commun,* 2021 Oct 8;12(1):5908; *Clin Cancer Res,* 2022 Jan 1;28(1):201-214). Here, we demonstrated the combination of OV-αCD47-G1 and temozolomide (TMZ) is a promising and practical strategy to improve BCBM treatment efficacy. We found TMZ treatment increased CD47 expression in both mouse and human breast cancer cells, while TMZ treatment alone had no adverse effect on OV-αCD47-G1 infection and had no effect on macrophage phagocytosis against breast tumor cells either. However, compared to OV-αCD47-G1 treatment alone, TMZ combination with OV-αCD47-G1 increased macrophage phagocytosis against breast tumor cells by enhancing the blockade of the CD47-SIRPα signaling pathway. Compared to OV-αCD47-G1 alone or TMZ alone, the combination of OV-αCD47-G1 and TMZ dramatically activated the transcription of typical macrophage cytokine genes in both human and mouse macrophages that were previously reported to respond to IgG1 antibodies, such as Il1β, Il6, Il12, Ccl2, Ccl4, and Nos2. TMZ combination with OV-αCD47-G1 also led to greater activation of human NK cell cytotoxicity through more effective binding to the Fc receptors when compared to treatment with OV-αCD47-G1 or TMZ alone. To evaluate the efficacy of OV-αCD47-G1 combined with TMZ for the *in vivo* treatment of BCBM, we utilized our previously described orthotopic model of human BCBM by i.c. injecting 1×10^5 firefly luciferase (FFL) gene-expressing human breast cancer cells (MBA-MD-231-FFL) into athymic nude mice (*Oncotarget*, 2016 May 10;7(19):27764-77). After tumor implantation, animals received an i.c. injection with OV-αCD47-G1, control OV, or saline as a placebo control with or without TMZ. OV-αCD47-G1 combined with TMZ improved survival of tumor-bearing mice better than OV-αCD47-G1 alone or TMZ alone. To avoid the cross-species interaction of human CD47 and mouse SIRPα, which is not physiologically relevant, we established a fully immunocompetent mouse model by replacing the anti-human CD47 antibody expressed from oHSV with anti-mouse CD47 antibody. Briefly, we constructed the anti-mouse CD47 antibodies (Clone: A4) on mouse IgG2b (equivalent to human IgG1) and mouse IgG3 (equivalent to human IgG4) scaffolds, termed as A4-IgG2b and A4-IgG3, respectively, as well as the corresponding oHSVs expressing A4-IgG2b (OV-A4-IgG2b) or A4-IgG3 (OV-A4-IgG3). Combination treatment with the mouse counterpart of OV-αCD47-G1, OV-A4-G2b, also enhanced mouse NK cell cytotoxicity, macrophage phagocytosis, and prolonged survival of mice bearing BCBM compared to the single treatments. The combination therapy enhanced the infiltration of macrophages, NK cells, and T cells compared to the single treatment in the tumor microenvironment. Collectively, our study indicates OV-αCD47-G1 combined with TMZ could improve immune-virotherapy for patients with BCBM. The translation of the study into the clinic, including IND-enabling studies, is ongoing.

296 Clinical Activity of Oncolytic Virus Voyager-V1 in Patients with Relapsed Refractory Lymphoma

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Voyager-V1 (VV1) is an oncolytic Vesicular Stomatitis Virus (VSV) encoding interferon beta and sodium iodide symporter (NIS) gene. Interferon beta confers tumor selectivity and enhances host antitumor immunity while the NIS gene allows noninvasive imaging of sites of viral infection using PET or SPECT. Mechanism of action of VV1 was established in early dose escalation studies showing infection of tumor cells through NIS imaging after intratumor injection of the virus. Immunohistochemical staining showed increase in immune cell infiltrates of injected tumors, supporting the rationale to combine VV1 with immune checkpoint antibodies. A number of clinical trials investigating safety and activity of VV1 are ongoing, utilizing VV1 as a monotherapy, or in combination with cemiplimab, an anti-PD1 antibody. Here, we report the results from our phase 1 clinical study evaluating the safety and tolerability of one intravenous (IV) infusion of VV1 in patients with relapsed refractory multiple myeloma and T cell lymphoma (TCL). This was a classical 3+3 phase I trial, starting at 5e9 TCID50 dose level 1 through 1.7e11 TCID50 dose level 4 given as a single IV dose. No dose limiting toxicities were observed. An expansion cohort at dose level 4 was added to enroll additional patients with TCL due to early signs of clinical activity. To date, 12 patients with relapsed refractory TCL have received one IV dose of VV1 at 1.7e11 TCID50. Two complete responses (CR) and five partial responses (PR) were recorded, with one CR lasting 30 months, and the other CR is ongoing at 15 months with no additional therapies. Adverse events were manageable, with transient decreases in lymphocyte counts and grade 1-2 cytokine release syndrome that is transient and resolving by 24h. Two cohort are added to the study to enroll 20 additional T cell lymphoma and 10 additional B cell lymphoma patients to obtain additional safety and efficacy signal and deeper analysis of biomarkers of responses.

297 Novel Oncolytic Therapy VET3-TGI Restricts TGFb1 and Augments Type-1 Immune Response in TME, Leading to Superior Therapeutic Efficacy in Multiple Preclinical Tumor Models

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Counteracting immunosuppressive TGFβ1 and enhancing Type-1 immune responses in tumor microenvironment, can lead to better anti-tumor immunity and durable clinical response. Current study developed a novel vaccinia virus known as VET3-TGI for oncolytic immunotherapy. VET3-TGI expresses soluble TGFβ mini-monomers, which acts as dominant negative inhibitor of TGFβ1 (TGFβi) and IL-12 to promote IFNγ/Type-1 responses in tumors. Additionally, CXCR3 was added to promote systemic delivery of VET3-TGI virus selectively to tumors and enhance tumor specific expression of therapeutic transgenes. The expression of transgene proteins: IL-12, TGFβi and CXCR3 was analyzed by ELISA, western and FACS. In vitro chemotaxis assays were used to analyze chemotactic response of CXCR3 to CXCR3 ligands. TGFβi was analyzed for their ability to alleviate TGFβ1 imposed suppression of proliferation and IL-12, for increased induction of GZMB/IFNγ in CD8 T cells. The therapeutic efficacy of VET3-TGI over control virus was tested in three pre-clinical *in vivo* mice tumor models (MC38, RENCA, EMT6), which demonstrated potent therapeutic activity, including 100% CRs, even at doses several logs below equivalent clinical doses. Post-mortem analysis in those tumor tissues showed that VET3-TGI showed reduced systemic toxicity and improved systemic delivery to tumors expressing CXCR3 ligands even in the face of pre-existing anti-viral immunity. Multiplex immunofluorescence analysis revealed the enhanced filtration of $CD3^+CD8^+$ T cell in tumors of VET3-TGI treated mice compared to control mice. RNA seq analysis of tumor samples showed VET3-TGI tumors showed profound changes of tumor microenvironment with polarization to type-1 immune response. Dramatic increase in IFNγ inducible genes: Type-1 inflammatory chemokines, interferon responsive factors, innate immunity, antigen presentation and concomitant decrease in TGFβ1 associated genes is evident in the analyses. Altogether, VET3-TGI demonstrated good ability to counter TGFβ1 mediated immunosuppression and dramatically enhance anti-tumor immune response leading to safe and potent therapeutic activity in multiple mouse tumor models. Based on the above, VET3-TGI is selected as the lead clinical candidate for upcoming clinical trials. Currently, clinical manufacture and toxicology testing is undergoing with a human version of this virus.

Preclinical Development of Novel Cell Therapies for Human Disease

284 Gene-Editing to Eliminate Automaticity Prevents Arrhythmias after Engrafting Human Pluripotent Stem Cell-Derived Cardiomyocytes

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Background: Pluripotent stem cell-derived cardiomyocyte (PSC-CM) transplantation is an emerging strategy to treat subacute myocardial infarction (MI), but transient engraftment-related arrhythmia (EA) is

major barrier to clinical translation. We hypothesize that EA is induced by pacemaker-like automaticity of developmentally immature graft. We initiated a CRISPR-Cas9 genome editing screen to identify the culprit ion channels. **Methods:** After testing single, double, and triple gene edits we found that knocking out the depolarization-associated genes *HCN4, CACNA1H,* and *SLC8A1*, combined with overexpression of the hyperpolarization-associated gene *KCNJ2,* resulted in PSC-CM that are electrically quiescent but excitable by external pacing. $150x10^6$ gene edited (n=3) and wildtype (n=7) PSC-CMs were transplanted into a minipig model of EA and observed for up to 7 weeks on continuous ECG for engraftment and arrythmia. **Results:** Genetically edited PSC-CM successfully engrafted and structurally coupled with host myocardium. No significant EA occurred after transplanting edited PSC-CM and all three subjects completed study. Low burden of isolated premature ventricular contractions (PVCs) and short episodes of non-sustained ventricular tachycardia (NSVT) occurred, but no sustained arrhythmias were observed. Heart rate and arrhythmia burden (36% vs 3% of the study duration) were significantly reduced ($p<0.001$). Conversely, wildtype PSC-CM controls demonstrated uncontrolled and sustained ventricular tachycardia (VT). Of the seven WT control subjects, four survived to study completion; three expired or were euthanized due to unstable VT or ventricular fibrillation. All grafts were structurally and electrically coupled with host myocardium. **Conclusion:** EA appears to be driven by an accelerated automaticity of early PSC-CM grafts. Genetically editing PSC-CM to abolish intrinsic pacemaker-like activity may prevent arrhythmia or at minimum, reduce arrhythmic burden and lethality. This study identifies ion channels involved in EA and supports a novel strategy to improve safety and tolerability of PSC-CM-based cardiac remuscularization therapy.

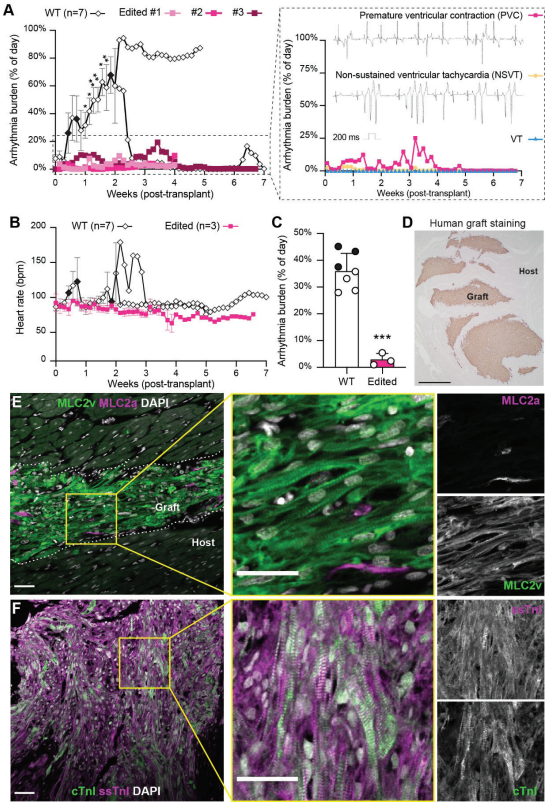

Figure. Edited PSC-CM do not cause significant arrhythmia following transplantation in minipig. (A-C) Arrhythmia burden and heart rate of minipigs following transplantation of edited PSC-CM. Black symbol denotes mortality/euthanasia due to unstable arrhythmia. Inset showing quantification of PVC, NSVT and VT burden in edited subject #3. **(D)** Representative edited PSC-CM graft at 7-weeks post-transplant, stained with human-specific β-myosin heavy chain. Scale bar = 1 mm. **(E, F)** Low and high magnification immunofluorescence images of edited PSC-CM grafts 4 weeks post transplantation marked by MLC2v/MLC2a and ssTnI/cTnI staining. Dotted lines indicate host/graft interface. Scale bars = 50 μm for low magnification; 20 μm for high magnification.

285 Engineering Stage-Specific Developmental Cues to Generate Non-Allogenic, iPSC-Derived CAR T Cells for Immunotherapy

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Introduction: Though chimeric antigen receptor (CAR) T cell therapy has produced remarkable results in treatment-refractory malignancies, the manufacturing process is challenging due to *de novo* generation of each therapy and variability in T cell phenotypes. A potential solution to this problem is the generation of CAR T cells *in vitro* from an infinitely renewing, allogeneic source of human pluripotent stem cells (hPSCs). There are two critical barriers to generating CAR T cells from hPSCs: 1. Expression of certain CAR transgenes early in T cell differentiation diverts development towards the innate lymphoid pathway instead of the conventional T lineage. 2. Removal of the endogenous T cell receptor (TCR) to prevent alloreactivity blocks development, as maturing T cells can no longer undergo positive selection. We addressed these barriers by deploying both an innovative gene editing strategy that allows stage-specific expression of transgenes, and a novel *in vitro* method to induce differentiation of mature conventional T cells. **Results:** The Artificial Thymic Organoid (ATO) is an *in vitro* method for generating mature, single positive (SP) CD8+ and SP4+ T cells from multiple stem cell sources including iPSCs. The ATO's ability to support positive selection from CD4+CD8+ (DP) precursors results in the robust production of mature, conventional T cells which carry a diverse repertoire of TCR expression on the cell surface (Fig 1A). We established a CRISPR-based gene editing strategy to achieve stage-specific expression of biologically active transgenes (e.g. CARs). Using this approach, we have shown: 1. Conventional T lineage production was blocked when a 2nd-gen CD19 CAR was expressed throughout differentiation. However, when the same CAR was placed under the control of the endogenous Granzyme A *(GZMA)* promoter, normal T cell differentiation in ATOs was unperturbed and mature, naïve CD45RA+CD62L+CD8α+CD8β+ CAR T cells were generated from T-iPSCs (Fig 1C). *GZMA* is expressed only in SP8/SP4 T cell and absent in earlier T stages. Resulting iPSC-derived CAR T cells

demonstrated antigen-specific cytokine response and proliferation. 2. To address alloreactivity, we ablated surface TCRαβ/CD3 expression by disrupting the T cell receptor alpha chain constant *(TRAC)* region (Fig 2A). We restored positive selection by transiently expressing an exogenous TCR driven by the *RAG1* promoter. *RAG1* is expressed during the double negative (DN) through double positive (DP) T cells but absent in SP8/SP4 T cells (Fig 1B). Thus, expression of the transgenic TCR occurred only during the critical period of positive selection (DP stage) and was absent after SP8/SP4 maturation (Fig 2B). In conjunction with CAR expression via *GZMA*, we thus generated mature, naïve TCRαβ-CD3- CAR T cells from T-iPSCs (Fig 2C). **Conclusion:** The developmental blocks that can occur during T cell differentiation from iPSCs as a result of genetic manipulation of CAR/TCR expression can be overcome through CRISPR-mediated, stage-specific expression of transgenes.

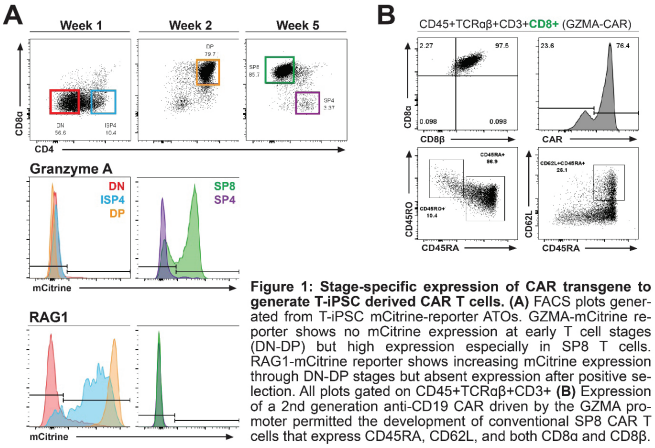

Figure 1: Stage-specific expression of CAR transgene to generate T-iPSC derived CAR T cells. (A) FACS plots generated from T-iPSC mCitrine-reporter ATOs. GZMA-mCitrine reporter shows no mCitrine expression at early T cell stages (DN-DP) but high expression especially in SP8 T cells. RAG1-mCitrine reporter shows increasing mCitrine expression through DN-DP stages but absent expression after positive selection. All plots gated on CD45+TCRαβ+CD3+ **(B)** Expression of a 2nd generation anti-CD19 CAR driven by the GZMA promoter permitted the development of conventional SP8 CAR T cells that express CD45RA, CD62L, and both CD8α and CD8β.

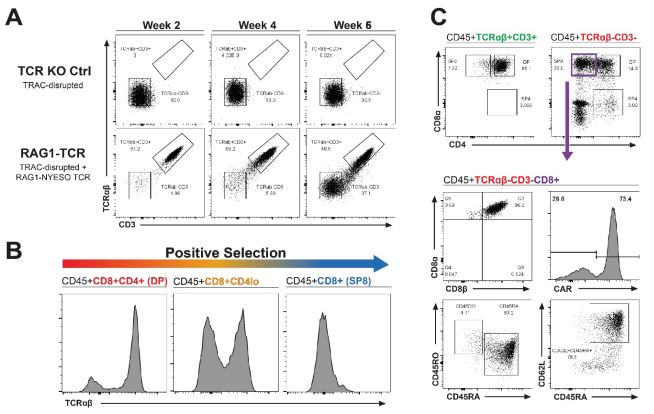

Figure 2: Transient expression of exogenous TCR rescues positive selection of T-iPSC derived CAR T cells. (A) Analysis of TCRαβ expression between TCR KO control and TCR KO + RAG-TCR T-iPSC ATOs. TCR KO was achieved by disrupting *TRAC* exon 1. All plots gated on CD45+. **(B)** Analysis of TCRαβ downregulation in TCR KO + RAG-TCR T-iPSC ATOs at week 6. As DP T cells undergo positive selection, TCRab and CD3 are downregulated and remain absent. **(C)** Analysis of SP8 CAR T cells from TCR KO + RAG-TCR + GZMA-CAR T-iPSCs at week 6. After positive selection, TCRαβ is downregulated and CAR is expressed using the GZMA promoter. Fully matured, TCRαβ -CD3- SP8s express naïve makers CD45RA, CD62L, conventional T markers CD8α, CD8β, and the 2nd generation anti-CD19 CAR.

286 Electrophysiological Maturation of Human Pluripotent Stem Cell-Derived Cardiomyocytes *via* Gene Editing

Silvia Marchiano[1], Kenta Nakamura[1], Hans Reinecke[1], Lauren Neidig[1], Michael Lai[2], Filippo Perbellini[2], Shin Kadota[3], Xiulan Yang[1], Jordan M. Klaiman[1], Lesley P. Blakely[1], Paul Fields[1], Elaheh Karbassi[1], Aidan Fenix[1], Lil Pabon[1], Lili Wang[4], Bjorn Knollmann[4], R. Scott Thies[2], Steven Kattmann[2], Nathan Sniadecki[1], W. Robb MacLellan[1], Alessandro Bertero[5], Charles E. Murry[6]

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Background: Automaticity, or the ability to spontaneously depolarize and fire action potentials (APs), characterizes the electrophysiological phenotype of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs), immature fetal CMs and mature pacemaker cells. The ion channels regulating AP formation are best understood in sinoatrial node CMs; however, whether those mechanisms apply to hPSC-CMs remains controversial. Moreover, arrhythmogenicity of transplanted hPSC-CM is a major barrier to safe clinical development of cardiac remuscularization therapy. We hypothesized that the hPSC-CMs' automaticity is modulated by developmentally regulated ion channel genes, and moreover, that genetic manipulation of these channels will result in hPSC-CMs that are quiescent-yet-excitable and thus safe for therapeutic use. **Methods:** To promote hPSC-CMs maturation we transplanted GCamP3-labeled hPSC-CMs into infarcted rat hearts as well as cultured them in vitro for up to 1 year. GCamP3-labeled graft were then isolated with laser-capture microdissection and we performed bulk RNAseq on both *in vivo* and *in vitro* samples at different time points. We analyzed the expression kinetics of all ion channels and used these to guide our CRISPR-Cas9 screening of ion channels involved in automaticity. **Results:** HPSC-CMs transplanted *in vivo* matured faster compared to *in vitro* long-term cultures, and the changes in genes involved in AP regulation reflect the presence of automaticity in immature hPSC-CMs. An extensive CRISPR screen revealed that the removal of pacemaker currents encoded by the genes *HCN4* and *CACNA1H*, the additional knockout of *SLC8A1* (encoding NCX1) and the addition of the hyperpolarizing potassium current I_{K1} (*KCNJ2*) yielded hPSC-CMs that are quiescent-yet-excitable. When transplanted *in vivo*, this quiescent-yet-excitable cell line electrically integrated with the host myocardium and beat in synchrony without inducing malignant arrhythmias. **Conclusion:** Systematic screening of ion channels revealed that automaticity in hPSC-CMs relies on both Ca^{2+} trafficking and voltage-membrane regulation. This study supports the concept that electrophysiological maturity of hPSC-CMs can be engineered through gene editing and be applied to cardiac remuscularization therapy.

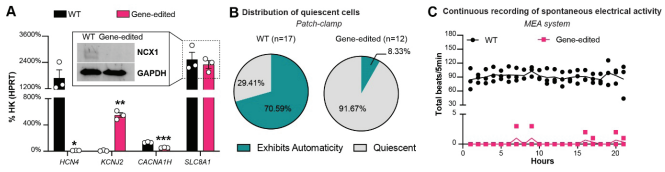

Figure 1. (**A**) RTqPCR and WB of ion channels targeted to remove automaticity in hPSC-CMs. (**B, C**) Patch-clamp and Multi-Electrodes Array (MEA) analysis showing decreased automaticity in gene-edited hPSC-CMs.

287 Induced Human Pluripotent Stem Cells Differentiation to Syndetome Informed by Single Cell Analysis

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Tendon injuries contribute to 45% of musculoskeletal consultations in the US alone and can be attributed to tendons' poor innate healing capacity. Besides invasive surgical intervention, to date there is no consensus on a biological or pharmacological approach for the healing process that is clinically practical. Bone marrow mesenchymal stromal cells and adipose stem cells have been explored as potential cell therapies, but they have limited self-renewal capacity and phenotypic heterogeneity. iPSCs have potential because they do not have these limitations. To date, there are very few protocols for tenogenic differentiation from human iPSCs, largely in part to the limited understanding of tendon development origins and how they differentiate from precursors. We sought to investigate a method of differentiation from human iPSCs to tenocytes that is more efficient and specific. Human iPSC lines were differentiated to Syndetome (SYN) in a stepwise manner through the presomitic mesoderm and somite (Fig1a). Successful differentiation was confirmed through significant upregulation or downregulation of known marker genes that characterize each step, along with IF (Fig1B-K). For further characterization, single cell RNA-sequencing (scRNA-seq) was conducted. From scRNA-seq analysis, we identified 11 distinct cell clusters (C0-C10), with clusters C3, C9, and C10 dominating the SYN stage (Fig1L). C3 expressed tenocyte markers, whereas C9 and C10 expressed neuronal markers. Trajectory analysis revealed that C3 is the main differentiation endpoint, but C9 and C10 in the later stages have branching off-target differentiation endpoints (Fig1M). IPA analysis shows that C9 and C10 can be characterized by increased Wnt activity leading to neuronal differentiation, whereas C3 shows decreased Wnt activity leading to tenocyte differentiation (Fig1N-O). Because Wnt family members were identified as being crucial in the generation of neural by-products, the addition of a Wnt pathway inhibitor Wnt-C59 (WNTi) was added to the later stages of the differentiation (Fig2A). ScRNA-seq post WNTi treatment revealed that the addition of WNTi not only dramatically increased the SYN population but also eliminated the side-products, resulting in a more specific cell population differentiating towards the tenogenic lineage (Fig2). In this study, we successfully differentiated iPSCs to tenocytes in a stepwise manner. ScRNA-seq trajectory analysis revealed off-target differentiation because of Wnt pathway activity. The addition of WNTi was used to address this, and further transcriptomics confirmed decreased off-target products and increased SYN/tendon specificity and efficiency. Elucidating the mechanism of the Wnt signaling pathway using a development-inspired protocol can lead to more powerful and specific differentiation protocols for cell therapy applications. iPSC-derive tendon progenitors can be an off-the-shelf cell source to tendon injuries that are currently untreatable or have poor surgical outcomes.

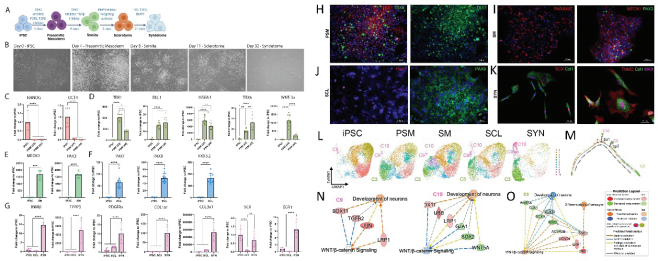

Fig.1. Gene expression analysis and ICC confirm successful differentiation from iPSC to Syndetome, however, scRNA-seq reveals off-target differentiation to neural-like progenitors. (A) iPSC to Syndetome stepwise differentiation protocol using chemically defined media and small molecules. **(B)** Micrographs of cells going through the differentiation stages at 20x. **(C)** Pluripotent markers are expressed in iPSCs and are downregulated in further stages. **(D)** Presomitic mesoderm markers are expressed at the PSM. **(E)** SM markers are upregulated at the SM. **(F)** Sclerotome markers are upregulated in the SCL stage. **(G)** Tenogenic markers are significantly upregulated compared to iPSC and SCL stages at the SYN stage. ICC staining shows expression of markers on the protein level: TBX1 and TBX6 of PSM **(H)**, PARAXIS, MEOX1, and PAX3 at SM **(I)**, PAX1 and PAX9 at SCL **(J)**, and SCX, COL1, TNMD, MKX at SYN **(K)**. Notable increase in cell size during the differentiation process was observed **(H-K)**. **(L)** UMAPS of each differentiation step show transition of 11 cell clusters from iPSC to SYN, in which the pluripotent clusters practically disappear, and 3 main clusters emerge: C3, C9, and C10. **(M)** Trajectory analysis based on the clusters shows that there are three main endpoints, with two of them off-target branching points. IPA analysis shows that C9 and C10 clusters, which are associated with neuronal differentiation, is linked with increased Wnt pathway activity **(N)**, while C3, which is associated with tenocyte differentiation, is linked with decreased Wnt pathway activity **(O)**.

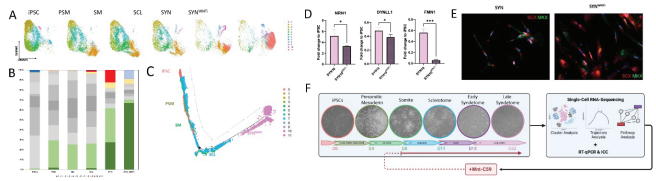

Fig.2. Addition of WNTi to the SCL and SYN induction stages of the differentiation increased SYN/tendon expression and removed off-targets. (A) UMAP comparison of the SYN and SYN^{WNTi} populations demonstrate increased size of C9, the SYN cluster, and elimination of C10, the neural cluster. **(B)** The proportion of cells in the SYN cluster (C0) has increased dramatically while the proportion of cells in the nueral cluster (C10) is removed after addition of WNTi. **(C)** Trajectory analysis reveals one main endpoint, the syndetome cluster. **(D)** There is significantly reduced expression of neural markers in the early syndetome stage with WNTi compared to without. **(E)** ICC demonstrates expression of SCX and TNMD (tendon markers) in both the SYN (left) and SYN^{WNTi} populations (right), but the SYN^{WNTi} cells appear to be more elongated and more robust. Images taken at 20x. **(F)** Informed by single cell transcriptomics, the addition of Wnt pathway inhibitor Wnt-C59 at the later stages of the differentiation resulted in a more specific differentiation of iPSCs to tenocytes.

288 Microglia Replacement with Bone Marrow-Derived Cells after Transient Inhibition of the Colony-Stimulating Factor 1 Receptor (CSF1R) is Superior to Standard Myeloablative Conditioning in Neuropathic Lysosomal Storage Diseases

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Allogeneic hematopoietic stem cell transplantation (HSCT) is the standard of care for several neurometabolic diseases. Recently, autologous transplantation of gene-modified hematopoietic stem and progenitor cells (HSPCs) showed significant benefits in clinical studies for severe leukodystrophies and neuropathic lysosomal storage diseases. The therapeutic effect of HSCT for these diseases relies on the ability of transplanted HSPCs to durably reconstitute the hematopoietic system and repopulate solid organs as tissue-resident myeloid cells in recipients pre-conditioned with myeloablative doses of Busulfan. The transplant of either donor-derived HSPCs or autologous gene-modified/edited HSPCs represents a promising approach to providing healthy cells and cross-correcting protein deficiencies in many neurologic and neurometabolic diseases. Despite advances in autologous approaches, the slow-paced and modest central nervous system (CNS) repopulation by bone marrow-derived cells remains

a key limitation to adequately address severe, rapidly progressing neurological diseases requiring prompt intervention. To address these limitations, we developed a novel conditioning regimen that enables fast, robust, and stable engraftment of HSPC-derived microglia-like cells in the CNS. The regimen combines Busulfan and PLX3397, a CSF1R inhibitor, which specifically depletes microglia/macrophages. In transplants of C57BL/6 mice with bone marrow (BM) from CAG:GFP/GFP mice, the combined regimen resulted in a 15-fold increase in the fraction of BM-derived GFP+ microglia-like cells in the brain compared to Busulfan alone (89±5% vs. 6±2.4% at 3 months, 86±4% vs. 23±17% at 7 months). The novel regimen also significantly accelerated the repopulation of the CNS. At early time points after BM transplant, the brain GFP+ fraction remained low with Busulfan (2.9±0.7%, 40 days) while it was substantial at 28 days (31±19%) and peaked at 40 days (92.7±1.5%) with PLX3397. Single-cell RNA-seq of brain-engrafted BM-derived and host CD45+CD11b+ cells showed tissue-specific cell signatures and a predominant homeostatic microglia-like phenotype. Normal hematopoietic reconstitution, neurobehavior, and the absence of brain inflammation confirmed that PLX3397 has no added toxicities. The addition of PLX3397 in xenogeneic transplants of human genome-edited CD34+ HSPCs in immunocompromised mice resulted in human cell chimerism in the brain without affecting their bone marrow engraftment or differentiation. To examine the combined regimen's potential in disease treatment, we used it to condition a mouse model of Mucopolysaccharidosis type I-Hurler (B6.129S Idua-W392X). We observed increased brain donor chimerism (3-fold) which resulted in a 4-fold increase in biochemical correction compared to Busulfan alone, confirming the superiority of this approach. Our findings provide comprehensive proof-of-concept of the efficiency and safety of a novel conditioning regimen to improve the efficacy and widen the application of HSCT for neurometabolic and neurological diseases.

289 Constitutive IL-7 Receptor (C7R) Signaling Enhances the Persistence of EBV-Specific T-cells

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Background: Almost 30% of Hodgkin's and Non-Hodgkin Lymphoma (HL and NHL) patients carry the Epstein-Barr Virus (EBV) genome in a type-2 latency state characterized by the expression of 4 viral type-2 latency antigens (T2-Ags)- EBNA1, LMP1, LMP2 and, BARF-1. Our lab and various others have used T-cells specific against these T2-Ags to target EBV-positive lymphoma with promising results in both autologous and allogeneic settings. However, there are challenges to enhancing the efficacy of EBV-specific T-cells (EBVSTs), including a lack of T-cell functional persistence in the suppressive tumor microenvironment (TME), attributed to a lack of cytokine support in-vivo. Interleukin-7 (IL-7), has both growth-promoting and anti-apoptotic effects on T-cells. However, limited IL-7 availability and downregulation of the IL-7 receptor in activated T-cells limit IL-7 signaling in-vivo. We previously showed that transgenic expression of a constitutively active IL-7 receptor (C7R) enhanced the persistence and anti-tumor efficacy of chimeric antigen receptors (CAR) T-cells in neuroblastoma and glioma xenograft murine model. Hence, we **hypothesized** that EBVSTs expressing constitutively active IL-7 receptor (C7R) to enable cytokine-independent proliferation will have increased persistence and potency in EBV+ malignancies. **Methods:** Expression of C7R was achieved by retroviral transduction of EBVSTs, which were further expanded with peptide libraries and cytokines to generate C7R-EBVSTs and unmodified EBVSTs. On day 16 post-expansion, EBVSTs were evaluated for their proliferation, antigen specificity and further in-vitro characterization. For in-vivo studies, we subcutaneously established EBV-transformed lymphoblastoid cell line (LCL) in NSG mice. EBVSTs were infused iv once the tumor reached a volume of ~130 mm^3. **Results:** EBVSTs expressing C7R (65%±25% transduction, n=6), were generated with stable specificity for T2-Ags (5064±2988 vs. 4683±2134 SFCs per 10^5EBVSTs) using an IFNg ELISpot assay; C7R-EBVSTs vs. unmodified EBVSTs). C7R-EBVSTs demonstrated antigen-specific cytotoxicity as measured by chromium release assay, enhanced STAT5-signaling (STAT5 MFI of 778±66 vs. 139±9 as measured by flow cytometric analysis) and enhanced in-vitro persistence in cytokine-starved cultures (35±2 days vs. 16±3 days for unmodified EBVSTs). C7R enhanced EBVST proliferation and decreased the frequency of Annexin V positive cells (indicating fewer apoptotic cells) in C7R-EBVSTs compared to unmodified EBVSTs when cultured in presence of TGF-beta, an immunosuppressive component of TME (14±2% vs. 26±3%. % Annexin V+ T-cells, n=3). In our murine model, mice receiving C7R-T2-EBVSTs cleared tumor by day 60, compared to day 240 ± 62 mm^3in response to unmodified EBVSTs; $p<0.000$, n=5/group). C7R-T2-EBVSTs also demonstrated enhanced persistence (67 days vs. 27 days for unmodified T2-EBVSTs). **Conclusion:** We successfully generated C7R-EBVSTs, which demonstrated enhanced antigen specificity and potency in-vitro and in-vivo. C7R provided protective effects against TgFb, an immunosuppressive component of the TME. Finally, the demonstration of enhanced T-cell persistence and potency along with enhanced STAT5 signaling provides a clinically translational platform. We are currently in the process of translating our findings to a phase I/II clinical trial against EBV-associated malignancies.

290 Stomach-Derived Human Insulin-Secreting Organoids for Diabetes Cell Therapy

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A leading strategy to reverse β-cell loss in Type 1 diabetes (T1D) and advanced T2D to achieve long-lasting normoglycemia is the transplantation of functional insulin-secreting cells. To overcome the shortage of human cadaveric islets, multiple approaches were developed to produce insulin-secreting organoids that mimic human islet function. We established a new technology for scalable production of pancreatic islet-like organoids from biopsy-derived human gastric stem cells (hGSCs) (manuscript under review at *Nature Cell Biology*, article available online at https://www.biorxiv.org/content/10.1101/2022.12.15.520488v1). These organoids contain gastric insulin-secreting (GINS) cells that resemble pancreatic β-cells and able to stably and durably reverse diabetes after transplantation. hGSC cultures can be reliably established from pinhead-sized tissues and expanded to 10^9 cells within 2 months, enabling both allogenic and autologous cell products. After sequential activation of the inducing factors NGN3 and PDX1-MAFA, hGSCs transitioned through a $SOX4^{High}$ endocrine and

a GAL^{High} GINS precursor before adopting the β-cell fate at efficiencies close to 70%. GINS cells were aggregated into islet-like organoids and acquired glucose-stimulated insulin secretion (GSIS) 10 days post differentiation. Notably, glucose-responsive GINS organoids can be produced from multiple donors. Moreover, GINS organoids responded to repeat glucose challenges as well as to the clinical anti-diabetes drugs Glibenclamide, Diazoxide, and GLP-2 analogues. scRNA-seq revealed that GINS organoids contained four endocrine cell types that closely resembled the four major human islet cells, namely, β-, α-, δ-, and ε-cells. Consistent with their functional competence, GINS cells expressed key genes involved in β-cell identity, metabolism, insulin synthesis and secretion, and ion channel activities. To characterize GINS cells in vivo, we transplanted GINS organoids under the kidney capsule of immune-compromised NSG mice. The grafts could live for more than 6 months and contained abundant insulin^{+} cells. Transplantation of 6 million GINS cells into STZ-induced diabetic mice rapidly reversed hyperglycemia and maintained glucose homeostasis for over 100 days until graft removal. Glucose tolerance was normalized in the rescued mice. We examined over 30 GINS grafts from one to six months post transplantation and found no evidence of EdU^{+} or Ki67^{+} proliferating cells, indicating that GINS organoids pose minimal tumorigenic risk. In summary, human GINS organoids have significant favorable attributes as a cell therapy product for insulin-dependent diabetes: (1) very low tumorigenic risk, (2) consistent and simple 3-step induction protocol, and (3) rapid acquisition of functionality in vitro. Ongoing work is focused on simplifying the manufacturing process and enhancing autoimmune resistance in the T1D setting.

Cell Therapy Product Engineering and Development | Non-Cancer

298 Immune Profiling of Respiratory Syncytial Virus (RSV) for the Development of Targeted Immunotherapy

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Background: RSV-associated respiratory viral infections are a major public health problem affecting the immunologically naïve/compromised population. For example, RSV-induced bronchiolitis and pneumonia are the leading cause of hospitalization in infants and young children worldwide, while in adult allogeneic hematopoietic stem cell transplant (HCT) recipients mortality rates as high as 80% have been reported in those in whom infections progress from the upper to the lower respiratory tract (LRTI). At the national level, the current (2022-23) RSV surge has resulted in a sharp rise in hospitalization rates, surpassing last year by almost 3-fold (https://www.cdc.gov/rsv/research/rsv-net). Our group has previously demonstrated the feasibility, safety and clinical efficacy of administering allogeneic ex vivo expanded multivirus-specific T cells (multi-VSTs) as a banked, off-the-shelf product for the treatment of EBV, CMV, BKV, HHV6, AdV and SARS-CoV-2 infections/disease in immunocompromised individuals. Given the RSV-associated morbidity and the paucity of preventative or therapeutic agents, we sought to characterize the cellular immune responses to RSV with a view to developing a targeted T-cell therapy product for those at highest risk of severe disease. **Methods:** To first identify immunogenic and protective RSV antigens we exposed PBMCs from healthy donors to pepmixes (overlapping peptide libraries) spanning the 11 RSV-encoded antigens of which 2 [Nucleoprotein (N) and Fusion (F)] were identified as immunodominant and advanced for VST manufacturing. We subsequently utilized our optimized VST manufacturing process and culture in a G-Rex device in medium supplemented with activating cytokines to generate RSV-specific VSTs (RSV-VSTs) with activity against these immunodominant targets. **Results:** We achieved a mean 5.2±1-fold increase in cell numbers (mean±SEM; n=13) that were comprised of CD3+ T cells (94.1±1.2%), with a mixture of cytotoxic (CD8+; 28.9±5.2%) and helper (CD4+; 71.1±5.2%) cells. These cells had a phenotype consistent with effector function and memory potential, as evidenced by upregulation of the activation markers CD25, CD69, and CD28 and expression of central (CD45RO+/CD62L+) and effector memory markers (CD45RO+/CD62L-) with minimal PD1, Tim3 or LAG3 expression. The antiviral activity of the expanded cells was confirmed by IFNγ ELISpot and all 13 lines proved to be reactive against the target antigens (N: 3587±939 SFC/2x10^{5}; F: 3526±743). As demonstrated by surface and intracellular flow cytometry, both CD4+ and CD8+ T cell subsets exhibited antiviral properties via IFNγ/TNFα production and upregulation of activation/costimulatory markers. Reactive cells were polyfunctional and primarily Th1-polarized as evidenced by IFNγ, Granzyme-B, IL-2 and GM-CSF production measured by FluoroSpot and Luminex array. Finally, the expanded cells were able to kill viral pepmix-loaded autologous PHA blasts with minimal/no activity against non-antigen-expressing autologous and allogeneic targets. **Conclusion:** Healthy donor-derived RSV-VSTs are Th1-polarized, polyfunctional and selectively able to kill viral antigen-expressing targets with no auto- or alloreactivity, indicative of their safety for clinical use. A clinical trial evaluating their use in HCT recipients as part of an off-the-shelf multi-respiratory virus-directed product is currently underway (NCT04933968, https://clinicaltrials.gov).

299 Co-Engineering Synthetically Programmed Cells and Biomaterials for Regenerative Medicine

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Introduction: A major objective of regenerative engineering is to overcome tissue degeneration and combat inflammation typical of pathologic states caused by injury, metabolic disorders, autoimmunity, and aging. One approach toward achieving these goals is delivery of biologic drugs, such as pro-inflammatory cytokine antagonists; however, the absence of means to reliably tune dosage leads to a compromise between treatment efficacy and adverse events. An alternative method is to transplant engineered tissue substitutes. One critical shortcoming of this approach is a lack of control over

transplanted cell behaviors. Here, we present a method combining cell engineering and biomaterial design to overcome such limitations. We leverage synthetic biology tools to create a customizable platform where cells engage materials to respond to dynamic features of the transplant niche with engineered, defined behaviors.**Materials and Methods:** Our system leverages the synthetic Notch (synNotch) receptor platform to program cellular responses to cues in their microenvironment. Like the native Notch receptor, synNotch is a juxtacrine receptor that regulates ligand-dependent transcriptional programs in cells. By exchanging Notch's extracellular domain with alternative recognition motifs and intracellular domain with synthetic transcription factors, we create synNotch receptors that produce user-specified sense/response behaviors. Since we can customize synNotch to drive expression of any transgene, we can tune defined cellular responses to selected orthogonal inputs, reducing crosstalk from native pathways. SynNotch, like native Notch, requires force on the order of piconewtons for activation. Thus, soluble monomeric ligands do not activate synNotch. We leveraged this feature to create biomaterials capable of capturing synNotch ligands, resulting in a privileged material:cell channel of communication, requiring co-localization of the engineered cell, the designer biomaterial, and the selected synNotch ligand. We termed our system MATRIX for material activated to render inducible expression. Here, we implemented MATRIX to enable engineered cells to respond to GFP, a bioinert cue, and activate cells for various regenerative functions.**Results and Discussion:** Deploying CRISPR epigenetic regulators could help modify the transcriptome in inflammatory environments. As a test of the potential for transcriptome modification, we used the MATRIX platform to potentiate effective knockdown of K-cadherin gene transcripts mediated by CasRx (Fig. 1A). Delivery of a TNF antagonist, soluble TNF receptor 1, initiated by MATRIX led to reduced inflammatory NF-κB pathway activation (Fig. 1B). Finally, MATRIX was able to induce pluripotent stem cells to differentiate into neurons as denoted by staining for the neuronal marker TUJ1 (Fig. 1C).**Conclusions:** The MATRIX platform is highly versatile with custom inputs and outputs. MATRIX represents an innovative approach to co-engineer cells and biomaterials for increased cellular and spatiotemporal control, as the requirement for co-localization of all components gates responses spatially. Continued development of the MATRIX platform will extend its capabilities into 3D hydrogels for eventual *in vivo* studies.

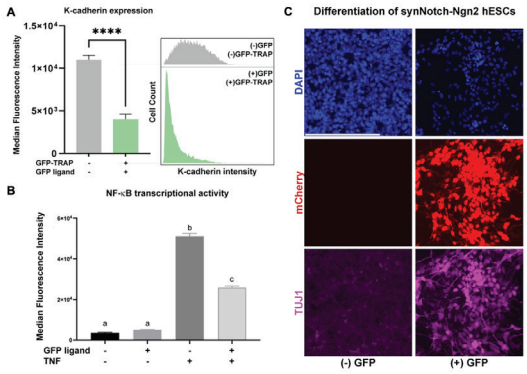

Fig. 1: A. Median fluorescence intensity of anti-K-cadherin immunolabeling demonstrating knockdown mediated by MATRIX induction of CasRx protein for transcriptome modification. (****p<0.0001, Student's t-test). B. Median fluorescence intensity of mKate2 reporter protein for the NF-κB transcriptional activity demonstrating effective immunomodulation of inflammatory environments through a TNF antagonist. Groups not sharing the same letters are statistically significant by two-way ANOVA with Tukey's post-hoc test. C. MATRIX platform-specific neuronal differentiation of embryonic stem cells with an mCherry activation reporter and anti-TUJ1 staining. Scale bar = 200 μm.

300 SEC-seq: Association of Molecular Signatures with Antibody Secretion in Thousands of Single Human Plasma Cells

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Protein secretion drives many functions in vivo; however, methods to link secretions with surface markers and transcriptomes have been lacking. By accumulating secretions close to secreting cells held within cavity-containing hydrogel nanovials, we demonstrate workflows to analyze the amount of IgG secreted from single human antibody-secreting cells and link this information to surface marker expression and transcriptional profiles from the same cells. Measurements using flow cytometry and imaging flow cytometry corroborated an association between levels of IgG secretion and CD138 expression. Using oligonucleotide-labeled antibodies and droplet-based sequencing, we show that pathways encoding protein localization to the endoplasmic reticulum, NADH complex assembly, and mitochondrial respiration were most associated with high IgG secretion. Altogether, this method links secretion information to cell surface and single-cell sequencing information (SEC-seq) and enables exploration of links between genome and secretory function. Our method lays the foundation for numerous discoveries in immunology, stem cell biology, and we envison applying this method to better engineer a cell therapy with high secretory therapeutic protein capacity.

301 Pancreatic Islet-Specific Engineered Treg Are Therapeutic in Mouse Models of Type 1 Diabetes

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Immunosuppressive regulatory T cells (Treg) have tremendous promise as a cell-based therapy for tissue-specific autoimmune disease. Importantly, antigen-specific Treg display greater therapeutic efficacy than polyclonal Treg in mouse models of autoimmunity. However, expansion of rare naturally occurring antigen specific Treg to clinical scale represents a major therapeutic challenge. To address this, we have devised strategies to generate antigen-specific engineered regulatory T cells (EngTreg) from CD4+ T cells using homology directed repair

(HDR)-based gene-editing of the *FOXP3* and *TRAC* loci, enforcing constitutive expression of the endogenous *FOXP3* gene while simultaneously replacing the endogenous T cell receptor (TCR) with a defined autoantigen-specific TCR. In previous work, we showed that antigen-specific EngTreg exhibit robust in vitro and in vivo suppressive function and achieve these functions independently of epigenetic events required to retain *FOXP3* expression in sorted Treg. In the current study, as a key step towards clinical application, we describe modeling of antigen-specific EngTreg function in animal models of type 1 diabetes (T1D). Using CD4+ T cells derived from transgenic non-obese diabetic (NOD) mice expressing the BDC2.5 islet-specific TCR, we generated mouse-equivalent islet-specific EngTreg by performing HDR-based gene-editing of the mouse *Foxp3* locus, inserting a strong promoter immediately upstream of the first coding exon to drive constitutive *Foxp3* expression. We modelled T1D by adoptively transferring either islet-specific monoclonal BDC2.5+ T effector cells (Teff) or polyclonal diabetogenic Teff isolated from hyperglycemic NOD mice into immunodeficient NOD-*scid*-IL2rγ^{NULL} (NSG) mice and demonstrated that these cells track to the pancreas and drive T1D in a manner dependent on T cell dose and clonality. Next, using these models, we demonstrate effective therapeutic intervention with EngTreg at both prevention and treatment timepoints corresponding to pre-diabetic and established disease states, respectively. Adoptively transferred BDC2.5 EngTreg homed to the pancreas and preserved islet β-cell integrity and insulin secretion. These data highlight both the direct and bystander suppressive capacity of antigen-specific EngTreg in the context of preventing or treating T1D.

302 New Generation Trained Mesenchymal Stromal Cell Therapy for Treatment for Rare Autoimmune Diseases

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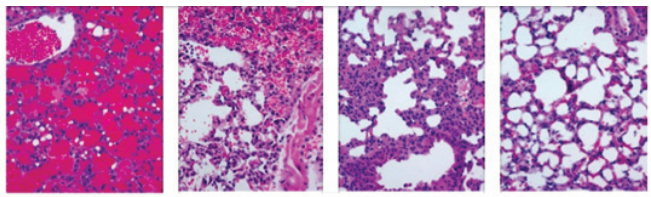
Background: Results from >100 clinical trials of cell therapy using untrained mesenchymal stem cells (MSCs) show that they are safe, but their clinical efficacy is variable.**Objectives:** We have developed a novel MSC-based cell therapy (HXB319) that potently reduces inflammation and halts organ damage. We show the efficacy of HXB319 in treatment of autoimmune diffuse alveolar hemorrhage (DAH).**Methods:** We engineered an MSC based cell therapy (HXB319) using a combination of cytokines and growth factors, and verified anti-inflammatory activity by flow cytometry, RT-PCR, and mass spectrophotometry. *In vivo* efficacy was assessed by treating autoimmune induced DAH in C57Bl/6 mice by intraperitoneal (IP) injection of pristane. Seven days later, some mice were treated with either MSCs or HXB319 (2X10^6 cells, ip). At sacrifice (day 14), peritoneal lavage fluid and lung tissue were sampled. **Results:** HXB319 cells showed significantly increased expression of anti-inflammatory genes (IDO and CD274), and markers of angiogenesis (CD146 and PDGFR-β). Protein pathway analysis showed significant upregulation of protein pathways that reduce inflammation (IL1RN, IDO), and fibrosis (with upregulation of HGF, c-MET signaling, while improving vascularization (VEGF). A pristane-induced, autoimmune, diffuse alveolar hemorrhage (DAH) model was utilized for proof-of-concept *in vivo* studies. In the DAH model, the mortality is typically above 75% beyond 14 days after pristane injection. *When delivered in vivo,* HXB319 reduced lung inflammation and significantly reduced alveolar hemorrhage (27.2%) vs. naked MSC treatment (75%) (Fig.1).



DAH DAH-MSC DAH-HXB319 C57B/6

Fig.1: Representative H and E staining of the pristane induced DAH model lungs, 20 x magnification

Pulmonary gene expression of IL-6, and serum IL-1 beta levels were only significantly reduced by HXB319 at day 14. Peritoneal lavage from mice treated with HXB319 showed reductions in total cells recovered, neutrophils, monocytes and NK cells compared to mice given MSCs or untreated DAH mice, but only the reduction in neutrophils was statistically significant (Table 1). Treatment with HXB19 cells significantly decreased the proportion of RORγT cells (Th17) in both CD4+ and CD8+ populations, and significantly increased the proportion of FoxP3+ cells among CD4+ cells (Tregs). Moreover, the proportion of CD4+ cells double-positive for RORγT and FoxP3 was significantly reduced.

Table 1: Splenocyte cell sorting

Cell Population	C57B/6	DAH	DAH-HXB319	DAH-MSC
PMN[a]	0.004 ± 0.001	0.53 ± 0.05*	**0.29 ± 0.10***	0.69 ± 0.10*
NK cells[a]	0.02 ± 0.01	0.10 ± 0.02*	0.05 ± 0.01	0.12 ± 0.03*
CD4$^+$ RORγT^{+c}	59.0 ± 0.66	83.3 ± 1.38*	75.0 ± 2.2*	77.8 ± 1.8*
CD8$^+$ RORγT^{+c}	59.7 ± 1.57	92.3 ± 0.78*	82.3 ± 3.4*	86.9 ± 2.0*
CD4$^+$ FoxP3^{+c}	0.29 ± 0.04	0.46 ± 0.67*	0.94 ± 0.15*	0.87 ± 0.10*
FoxP3$^+$ / RORγT^{+d}	0.48 ± 0.07	0.56 ± 0.09	1.27 ± 0.05*	1.14 ± 0.15*

Conclusions: Novel HXB319 cell therapy, phenotypically engineered to control inflammation, is a potent anti-inflammatory, anti-fibrotic, and angiogenic allogeneic cell therapy. A single dose may suppress severe autoimmune disease activity, such as pulmonary hemorrhage.

303 Human Chimeric Antigen Receptor-Mesenchymal Stem Cells (CAR-MSCs) Display Enhanced Trafficking and Immunosuppressive Efficacy

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Allogeneic mesenchymal stromal cells (MSCs) are widely studied for their potential as a cell therapy treatment for autoimmune diseases due to their inherent homeostatic capabilities in the body. Although clinical trials have demonstrated the safety of MSC therapy, their therapeutic efficacy in autoimmune disease treatment remains insufficient due to two major limitations: **1)** poor **trafficking** of MSCs to target tissues and **2)** suboptimal i**mmunosuppressive** activity by MSCs following their ex vivo culture. We hypothesized that chimeric antigen receptor mesenchymal stromal cells (CAR-MSCs) could be created for the treatment of autoimmune disease based on CAR construct design. Here, we predicted that trafficking could be improved by a **1) single chain variable fragment** (scFv) designed for binding to target tissues of interest fused to **2) intracellular signaling domains** designed to promote immunosuppressive enhancement of MSCs. As a proof of concept for CAR-MSC therapy, we created CAR-MSCs directed to E-cadherin (Ecad) expressed on epithelial colon tissues susceptible to immune cell attack especially in etiologies such as graft-vs-host disease (GvHD) and irritable-bowel-disease. We hypothesized that anti-Ecad CAR-MSCs (EcCAR-MSCs) with human and mouse Ecad cross-reactivity and a CD28ζ intracellular signaling domain would induce trafficking and antigen-specific immunosuppression at these inflammatory target tissues. We transduced CAR into adipose-derived MSCs via lentiviral vector enhancement creating CAR-MSCs and tested both their immunosuppressive capabilities and trafficking to the colon *in vivo*. To test CAR-MSC **immunosuppressive efficacy**, GvHD xenograft models were induced via intravenous injection of human peripheral blood mononuclear cells into NOD-SCID-$\gamma^{-/-}$ mice. Mice were treated by intraperitoneal injection of CAR-MSC, untransduced (UTD-MSC), or vehicle control. We found that CAR-MSC treatment in these mice prevented their weight loss (**a**), decreased their clinical GvHD score (**b**), increased human T cell suppression (**c**) pushed T cells towards a Treg-like phenotype (**d**), and improved overall survival as compared to controls (**f**). Antigen-specific stimulation of CAR-MSCs were evaluated by RNAseq analysis of stimulated and unstimulated CAR-MSCs and UTD-MSCs. Here, a distinct upregulation of immunosuppressive gene signatures was found in Ecad stimulated CAR-MSCs only (**g**). Finally, study the impact of scFv specificity on the **trafficking** and treatment efficacy of CAR-MSCs, we employed our GvHD xenograft model and compared the effects of Luciferase^{+} CAR-MSCs with a randomized control scFv to our anti-Ecad scFv CAR-MSCs. We found that anti-Ecad CAR-MSCs led to a significant prevention of GvHD induced weight loss (**h**) and that CAR-MSCs with an anti-Ecad scFv preferentially trafficked to the colon in our mouse models (**i**). In summary, our findings introduce CAR-MSCs as a novel therapeutic platform to enhance MSC antigen-specific immunosuppression and trafficking for improved treatment outcomes in GvHD and other autoimmune diseases.

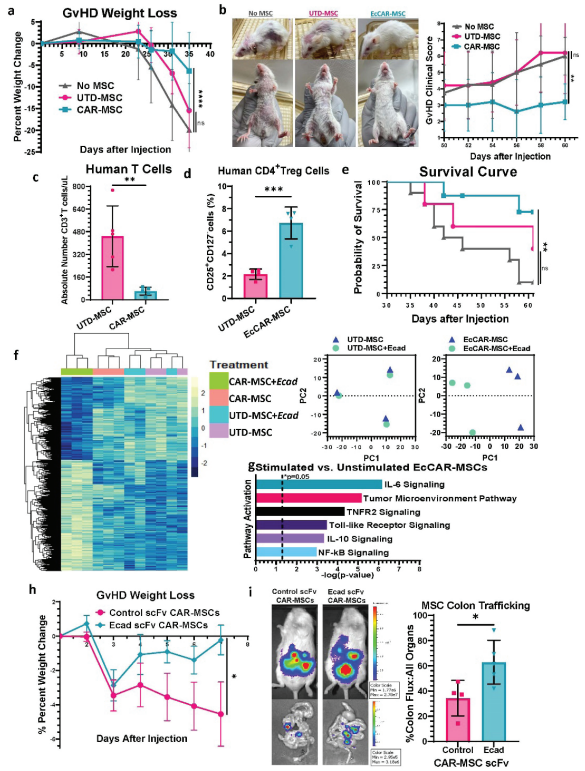

Fig. 1| CAR-MSC vs. UTD-MSC treatment in GvHD mouse model induced by human PBMCs injection into NSG mice displaying **a)** %bodyweight loss across treatment groups **b)** Clinical GvHD scoring across groups with symptoms including diarrhea, posture, activity, fur and skin integrity **c)** Number of human CD3^{+} T cells/uL in the peripheral blood of CAR-MSC vs. UTD-MSC treated mice **d)** %CD4^{+}CD25^{+}CD127^{-} T cells identified in cell populations **e)** Survival outcomes across GvHD treatment groups **f)** Gene expression profiles by RNAseq of stimulated and unstimulated CAR-MSCs vs. UTD-MSCs where UTD-MSCs cluster by MSC donor, while CAR-MSCs cluster by stimulation status. **g)** Gene set enrichment analysis of stimulated vs. unstimulated CAR-MSCs **h)** %bodyweight loss across mice receiving Luciferase^{+} anti-Ecad scFv CAR-MSCs vs. random control scFv CAR-MSCs with **i)** CAR-MSC flux ratio of trafficking to the colon. (ns=p≥.05, *p≤.05, **p≤.01, ***p ≤ .001, ****p≤.0001 by t-test, ANOVA, or Kaplan survival analysis, n≥3 MSC donors)

304 Engineered Microenvironment Converters (EM-Cs): Macrophages Expressing Synthetic Cytokine Receptors Rebalance Pro-/Anti-Inflammatory Signals in Disease Microenvironments

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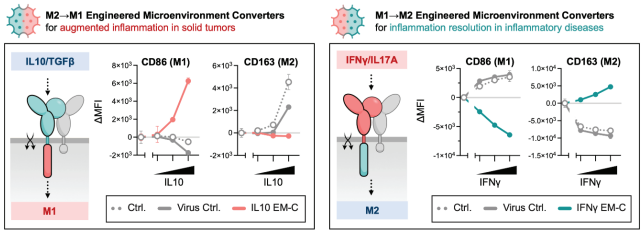
Background: Cytokines mediate the balance of pro- and anti-inflammatory signals in tissue microenvironments. Dysregulated or imbalanced cytokine expression supports numerous diseases by promoting deleterious immunosuppression or inflammation. As examples, anti-inflammatory cytokines found in solid tumors suppress immune activation and safeguard the tumor, whereas pro-inflammatory cytokines in fibrotic tissues drive chronic inflammation. Rebalancing inflammation/immunosuppression by rectifying cytokine signals thus offers a flexible strategy for treating many diseases. However, doing so through traditional cytokine blockades is besieged by safety risks due to systemic effects. Macrophage-based immunotherapies instead offer the potential to localize to diseased environments, detect pathogenic cytokines, and proportionately rebalance inflammation as needed. Here,

we engineered macrophages with synthetic cytokine switch receptors (SR) to develop a cell therapy platform for modulating pro-/anti-inflammatory signals. Macrophages are homeostatic regulators capable of initiating and resolving inflammation, and we leveraged this natural proficiency using SRs that convert tumor-related immunosuppressive (M2) signals into pro-inflammatory (M1) responses for tumor microenvironment (TME) modulation, or vice versa for inflammatory diseases. We termed this platform "Engineered Microenvironment Converters" (EM-C) and evaluated its modular ability to target several disease-associated cytokines. **Methods:** EM-Cs targeting IL10, TGFβ, IFNγ and IL17A were generated by expressing SR in primary human macrophages and monocytes. M2-to-M1 SR were designed to convert IL10 or TGF-β into pro-inflammatory stimuli, and M1-to-M2 SR were designed to IFNγ or IL17A into immunosuppressive signals. The in vitro response of EM-Cs to their target cytokine was monitored using phenotypic characterization of surface molecules, measurement of cytokine release, mRNA profiling, and biochemical analysis of downstream signaling. Co-culture assays with bystander cells were used to assess the ability of EM-Cs to alter their microenvironment. **Results:** Pro-inflammatory EM-Cs converted IL10 and TGFβ, two prevalent immunosuppressive cytokines in the TME, into pro-inflammatory signals by upregulating M1 markers, cytokines, and pathways in a dose-dependent manner. EM-Cs furthermore repolarized bystander M2 macrophages towards a pro-inflammatory phenotype following co-culture. Anti-inflammatory EM-Cs converted IFNγ and IL17A, two cytokines canonically overexpressed in inflammatory disease, by upregulating M2 markers and suppressing cytokines and pathways associated with chronic inflammation.**Conclusion:** We present a novel immunotherapy platform that harnesses macrophages as "living converters" to locally regulate cytokine imbalance for oncology and inflammatory applications. We demonstrate EM-Cs in the M2-to-M1 and M1-to-M2 direction, showcasing a modular ability to control the inflammatory status of microenvironments without systemic cytokine antagonism.



Gene Targeting and Gene Correction: Hemoglobin, Muscle, and Eye

305 Exceptional Fetal Hemoglobin Induction by *BCL11A* +58/+55 Combined Enhancer Editing and Hydroxyurea Treatment in Rhesus Monkeys

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β-hemoglobinopathies are the most common monogenic disorders with significant morbidity and mortality. Elevated fetal hemoglobin (HbF) expression can mitigate the phenotype and CRISPR-Cas9 mediated editing of HbF repressors in autologous hematopoietic stem/progenitor cells (HSPCs) has emerged as a promising new therapeutic intervention. Early data from clinical trials targeting the +58 DNase region of the erythroid-specific enhancer of the *BCL11A* gene are promising, yet variability in the HbF response led us to speculate that combined editing of the +58 and +55 enhancers could maximize the effect. Combined editing with 3×NLS-SpCas9 protein and 2 sgRNAs in rhesus CD34+ HSPCs provided higher mean levels of HbF (81.9%) among erythroid progeny than single-edited (~55%) and non-edited control groups (18.3%) without affecting enucleation or colony-forming unit ability. Next, we transplanted 3 rhesus macaques with CD34+ HSPCs following combined editing after confirming HbF reactivation (70.1 - 95.8% in edited *vs* 7.3 - 20.6% in control groups) and editing frequencies (88.6 - 98.1%) *ex vivo*. Busulfan-conditioned animals transplanted with edited cells recovered with typical reconstitution kinetics and displayed long-term stable editing frequencies (91.7 - 95%) among peripheral blood (PB) granulocytes (127 - 200 weeks post-infusion). Animals displayed high levels of F-cells (51 - 93%) and γ-globin (32 - 75%) early post-transplantation which gradually reduced and stabilized to 27 - 52% and 13 - 38%, respectively. Along with control animals, two of the HbF stabilized editing animals were phlebotomized to induce stress erythropoiesis. An absolute increase in F-cells (17% and 33%) and γ-globin expression (12% and 20%) over stable levels were noted for only edited phlebotomized animals. Further, phlebotomized animals were treated with an HbF inducing agent, hydroxyurea (HU), that provided an additional absolute increase in F-cells (13% and 16%) and γ-globin (14% and 15%) levels in only edited animals. The augmented levels came back to stabilized levels after stopping both phlebotomy and HU treatments. Administering HU without phlebotomy to the 3rd edited animal along with a control animal also led to a significant absolute increase in F-cells (26%) and γ-globin (16%) levels in the edited animal with no change noted in the control. Editing analysis among sorted cell lineages from PB and

bone marrow (BM) including erythroid progenitors and CD34+ HSPCs revealed high levels of editing except CD3+ T-cells, probably due to relatively less lymphotoxic busulfan conditioning. Further clonal analysis demonstrated that only a small portion of the clones was unedited (<8%) and the remaining clones were mutated by roughly equal contributions of indels, programmed inversions and deletions (n= 232 - 272). Additionally, most of BM cells displayed disrupted core enhancer $TGN_{7\text{-}9}WGATAR$ half E-box/GATA binding motifs (n= 399 - 544). In summary, combined editing of the *BCL11A* +58 and +55 enhancers provides clinically relevant HbF levels which can be augmented pharmacologically to maximize the HbF response. Combined edited cells displayed long-term durability (~4 years) with sustained HbF induction without hematologic disturbances and may serve as a long-term solution for patients with β-hemoglobinopathies.

306 EPI-321, a Potential Cure for FSHD

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most prevalent skeletal muscular dystrophies. FSHD is characterized by DNA hypomethylation of D4Z4 repeat units of a macrosatellite array found at the distal end of chromosome region 4q35, which causes a myotoxic expression of DUX4, disrupting numerous signaling pathways that converge on muscle cell death. DUX4 expression is stochastic, which complicates the development of drugs to inhibit its protein expression. There is no cure for FSHD and current standard of care focuses on managing the symptoms to improve patient quality of life. At Epic Bio, we leverage our proprietary GEMS (Gene Expression Modulation System) platform to develop a treatment for FSHD that targets the D4Z4 epigenome and to permanently suppress DUX4 expression. Our product, EPI-321, is an AAV serotype rh74 vector encoding a catalytically inactive Cas protein fused to gene-suppressing modulators, under the expression of the CK8e promoter and a guide RNA targeting D4Z4. EPI-321 was shown to robustly suppress DUX4 expression and downstream cascade *in vitro* in FHSD patient derived myocytes, indicating proper binding on D4Z4 and subsequent epigenomic silencing. Next, the functionality of EPI-321 in patient-derived myocytes was determined by examining their apoptotic rate upon treatment with NucView® Caspase 3/7 dye. A significant decrease and return to normal myocytes apoptotic levels was observed. Finally, to evaluate *in vivo* efficacy of EPI-321, we examined the FSHD cell content 24 days following one-time intravenous administration of EPI-321 in a humanized murine model of FSHD. In this model, EPI-321 resulted in a phenotypic rescue with 55% increased survival of skeletal muscle cells. Taken together, these data support the development of EPI-321 as a one-time gene therapy treatment for FSHD patients. We plan to file an IND by year end 2023, and anticipate initiating first-in-human trials in 2024.

307 Efficient Engraftment of Genome Edited CD34+ HSPCs in CD45 Antibody-Drug Conjugate (ADC) Conditioned Non-Human Primates

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Sickle cell disease (SCD) is the most common monogenic inherited disorder that affects red blood cells (RBC) and leads to acute and chronic complications including pain crises, strokes, end organ damage and early mortality. As sickle cell patients with mutations resulting in persistent postnatal elevated pancellular fetal hemoglobin ($\alpha_2\gamma_2$, HbF) expression have ameliorated disease severity, autologous transplantation with hematopoietic stem and progenitor cells (HSPCs) edited with CRISPR-Cas9 to inactivate the HbF repressor *BCL11A* erythroid enhancer is under development for treatment of SCD and β-thalassemia. While early clinical trials show promising results, morbidity and mortality due to cytotoxic chemotherapy-based pre-transplant conditioning are significant barriers. We developed a single-dose anti-CD45 antibody-drug conjugate (MGTA-45 ADC) conditioning regimen targeting blood cells including hematopoietic stem cells (HSCs) with minimal off-target toxicities. MGTA-45 robustly killed primary human HSCs and peripheral blood (PB) mononuclear cells (MNCs) (5.8-6.6 pM, n = 4) *ex vivo*. In humanized mice, a single dose of MGTA-45 (1 mg/kg, n = 5) achieved >95% depletion of human HSCs in bone marrow and immune cells in PB and bone marrow while isotype-ADC had minimal effect. In cynomolgus macaques, a single dose of ≥0.15 mg/kg MGTA-45 resulted in >95% bone marrow CD34+/CD90+ HSC and broad peripheral immune depletion (n = 3-6 per dose). MGTA-45 had rapid *in vivo* clearance ($t_{1/2}$ < 1.2 hour at 0.2 mg/kg), facilitating use in transplantation. For transplant studies, rhesus macaque CD34+ HSPCs were electroporated with 3×NLS SpCas9 protein and two RNAs targeting the +55 and +58 *BCL11A* erythroid enhancers. The edited cells showed editing of 93.15 - 98.65%, with γ-globin expression of 82 - 89% following erythroid differentiation compared to controls with 10 - 31% γ-globin. The edited HSPCs were autologously infused 10 days after MGTA-45 administration. At 0.2 mg/kg MGTA-45 (n=1), editing reached 62% of target alleles among short-lived granulocytes, reflecting the HSC compartment, with a follow up of 72 weeks to date. At 0.3 mg/kg MGTA-45 (n=2), granulocyte editing reached 82 - 84% and remained stable through 24 and 32 weeks to date. γ-globin expression and F-cell levels reached 67 - 82% and 80 - 93% in these animals respectively, at week 10-13, comparable to control animals receiving dual edited cells after conditioning with myeloablative busulfan at 5.5 mg/kg × 4 days (n=3). Editing in other lineages including monocytes, NK cells, B cells, erythroid precursors and CD34+ HPSCs ranged from 79-93% for the animals given 0.3 mg/kg MGTA-45 and 34-63% for the animal given 0.2 mg/kg MGTA-45. At

16 - 69 week post-transplantation, T cell editing was 14-21% following MGTA-45 conditioning (8-31% for busulfan treated animals at 25 - 34 weeks). We conditioned two additional animals with 0.2 mg/kg MGTA-45 and infused autologous HSPCs transduced with a barcoded lentiviral vector. At 4 - 8 months follow-up, the animals are highly polyclonal, similar to TBI or busulfan conditioned animals. In summary, MGTA-45-based conditioning is myeloablative and achieved polyclonal early engraftment at levels equivalent to TBI or myeloablative busulfan. This approach allowed robust engraftment with *BCL11A* enhancer edited HSCs and achieved potentially therapeutic levels of HbF. These data support the use of MGTA-45 conditioning for diverse transplantation applications.

308 *In Vivo* Genetic Eye Disease Correction Using Split AAV-Mediated Adenine Base Editing

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Purpose: Stargardt disease (STGD) is an inherited blinding disorder. The vision loss in STGD patients is associated with the toxic build-up of lipid-rich deposits in the retinal pigment epithelial (RPE), which supports and nourishes the retinal photoreceptors, primarily cones. STGD is caused by mutations in the *ABCA4* gene. Currently, there is no approved therapy. The most frequent variant c.5882G>A, p.G1961E, a G to A point mutation which affects approximately 15% of STGD patients, is a potential target for adenine base editors (ABEs). We developed a dual adeno-associated viral (AAV) vector split-intein adenine base editing strategy to correct the *ABCA4* (c.5882G>A, p.G1961E) gene. **Methods:** To develop the base editing strategy, we designed and tested different guide RNAs (gRNAs) in combination with the ABEs to target the c.5882G>A mutation in an *ABCA4*^{1961E} HEK293T cell line which carries the mutation on a lentivirus insert. To assay base editing in relevant target cells (photoreceptors and RPE cells), we developed an engineered human retinal organoid model and humanized mouse model carrying the *ABCA4* c.5882G>A mutation. We identified a gRNA to show the highest efficiency at the c.5882G>A target base at position 7 (A7) inside the base editing window. Importantly, there is an adjacent adenine (A8) at the 3rd position in codon 1961 (wobble base) that can also be edited at similar efficiencies and does not affect the amino acid sequence (silent change). This wobble base (A8) can be used as a surrogate assay in models which lack the c.5882G>A mutation. Throughout the study, we analyzed A7 target base editing (*ABCA4*$^{1961E/E}$ retinal organoids, and humanized *ABCA4*$^{hu1961E/G}$ mice) and A8 wobble base editing on wild-type human retinal explants and in RPE/choroidal explants. Finally, we subretinally injected adult cynomolgus macaques and assayed base editing rates of the bulk genomic DNA, bulk RNA, and on the DNA from sorted rods and cones. **Results:** Base editing efficiency was similar at the Stargardt target base (c.5882G>A) and the adjacent silent base (c.5883A) in mutant models (i.e., lentivirus-carrying HEK293T cells and *ABCA4*$^{1961E/E}$ retinal organoids). Our split-intein adenine base editor AAV vector led to editing at the A8 base of 5-15% in retinal organoids and 10-20% in human retinal explants, assayed at the genomic DNA level. We also injected the optimized vector construct into mutation carrying mice and achieved high *in vivo* gene correction, where ~50% of photoreceptors and ~80% of RPE cells showed editing. In injected macaques, base editing rates were found to be ~50% in cones, ~25% in rods and ~70% in RPE cells. To analyze genome-wide off-target effects of our base editing strategy on human retinal explants, we performed targeted deep sequencing and no off-target base editing was identified. **Conclusions:** We have developed a highly effective and precise base editing strategy for Stargardt disease for the correction of the *ABCA4* (c.5882G>A, p.G1961E) mutation in various mouse, human and macaque models. We not only show gene editing in primate photoreceptors and RPE cells but demonstrate the ability to correct the disease-relevant mutation in retinal organoids and mice. These results demonstrate that AAV-mediated base editing can lead to a precise genetic change in the retina in vivo.

309 *In Vivo* HSC Base Editing for Gene Therapy of Sickle Cell Disease in a Mouse Model

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Sickle Cell Disease (SCD) results from a single nucleotide transversion (A to T) in the sixth codon of the β-globin gene. Precise genome engineering in hematopoietic stem cells (HSCs) represents a potential curative option for SCD patients. We aimed to correct the SCD phenotype by converting the sickle mutation (GTG) into a benign G-Makassar variant (GCG) using in vivo base editing in HSCs. We have developed a next-generation gene therapy approach involving *in vivo* transduction of mobilized hematopoietic stem cells. The central idea of this *in vivo* approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus (HDAd) gene transfer vector system. Transduced cells return to the bone marrow where they persist long-term. For efficient HDAd vector production, we utilized miRNA-regulated gene expression, virus associated RNA (vaRNA), and anti-CRISPR inhibitors to suppress transgene expression in HDAd producer cells. HDAd vectors expressing the latest version of an adenine base editor (ABE8e) linked with a Cas9 variant (NRCH) under a human PGK or EF1α promoter were successfully produced. *Ex vivo* transduction of lineage-minus (Lin$^-$) cells from SCD mice (Townes/CD46) with HDAd-PGK-Maka or HDAd-EF1α-Maka followed by transplantation into irradiated recipient mice led to 55.2% to 88.2% stable correction of the sickle cell mutation (GTG>GCG; V6>A) in peripheral blood cells. The HDAd-EF1α-Maka was chosen for downstream experiments due to its higher activity. In an *in vivo* HSC transduction setting, involving HSC mobilization by G-CSF/AMD3100, intravenous injection with HDAd-EF1α-Maka, and short-term selection with low doses of

O^6BG/BCNU, an average of 23% (range 17-35%) correction of the sickle mutation was achieved. Deep sequencing of week 16 bone marrow samples revealed that most of the edited alleles contained the expected GTG>GCG conversion. Less than 1.5% byproducts (mostly short deletions) around the guide RNA-targeting region were found, consistent with the property of base editors to function independently of double-stranded DNA breaks. Phenotypic correction was observed in treated Townes/CD46 mice, including reticulocyte counts, spleen sizes, hematological and histological parameters. We did not observe significant adverse effects related to *in vivo* transduction/selection. Lin^- cells were isolated from *in vivo* transduced animals and plated for colony-forming unit (CFU) assay as well as transplanted into irradiated C57BL6 recipient mice. The CFU assay showed that 19.2% and 12.5% of colony-forming stem cell progenitors had monoallelic and biallelic correction, respectively. The editing levels remained stable at week 12 after transplantation (ongoing). In $CD34^+$ cells from a SCD patient, transduction with HDAd-EF1α-Maka led to 11% GTG>GCG conversion at the sickle mutation site. This is significantly higher than the 4.5% correction level with a prime editing strategy (HDAd-PE5max) reported previously. We are currently conducting studies in SCD $CD34^+$ cells from additional donors. Our *in vivo* approach requires only one non-integrating vector, only intravenous/subcutaneous injections, and minimal *in vivo* selection. This technically simple approach has the potential for application in resource-poor countries where SCD is prevalent.

310 Optimization and Characterization of Genome Editing of Human Hematopoietic Stem Cells to Induce Fetal Hemoglobin towards SAGES-1 Clinical Trial

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Sickle cell disease (**SCD**) is a severe blood disorder caused by a point mutation in the *HBB* gene. One promising genome editing strategy to treat SCD is to reactivate anti-sickling fetal hemoglobin (HbF). Results of ongoing clinical trials targeting the erythroid specific enhancer of BCL11A to induce durable levels of HbF are encouraging. We targeted alternative downstream repressor-binding sites in the γ-globin gene promoters and identified -115 site as more potent inducer of HbF (32% ± 1.5) compared to -196 *ZBTB7A* repressor binding site (16.8% ± 0.9) in erythroid progeny of $CD34^+$ transplanted *NBSGW* mice. Here we describe IND-enabling studies to advance a planned St. Jude Autologous Genome Edited Stem Cell (SAGES-1) clinical trial. To evaluate the efficacy of -115 γ-globin promoter editing, we edited plerixafor-mobilized $CD34^+$ HSPCs from one healthy donor and three individuals with SCD and transplanted them into *NBSGW* mice. We attained high-frequency editing in $CD34^+$ HSPCs before (85.6% ± 0.9) and after 17 weeks of transplantation (74.2% ± 2.9) that resulted in strong HbF induction (27.6% ± 1.7) in red cell progeny derived from mice bone marrow. Single-cell western blots revealed that 49.3% to 58.2% of edited erythroblasts exhibited γ-globin expression compared to unedited controls (<6%). By single-cell RNA-sequencing, we noticed similar upregulation of γ-globin transcripts in edited erythroblasts. Importantly, functional effects of broad HbF induction assessed where edited cells exhibited three-fold reduction in sickling under hypoxia. To assess associated genotoxicities, we applied CHANGE-seq to discover and rhAMP-seq to validate genome wide off-target activity. We observed no detectable off-target mutations above background at 194 candidate nominated off-target sites. We further characterized genomic re-arrangements by Uni-Directional Targeted Sequencing (UDiTaS) and noted no recurrent translocations at day 5 and 14. By long-range PacBio sequencing analysis, we observed a dominant 4.9kb deletion resulted from simultaneous double strand beaks in *HBG1* and *HBG2* promoters. We further confirmed by digital droplet PCR 29.8 % ± 1.3 of 4.9 kb deletions observed in edited $CD34^+$ HSPCs. To produce genome editing components required for autologous cellular therapy, we optimized a tag-free purification method to generate GMP-grade Cas9-3xNLS with high purity (>99.7% by RPLC) and low endotoxin. Potential guide RNA contaminants were identified by a modified gRNA sequencing assay where we observed 82.2% to 84.2% of the 5' protospacer sequence perfectly matches to the targeted gRNA sequence with no detectable contaminants from unrelated sequences. We optimized editing of healthy donor $CD34^+$ HSPCs at clinical scale using Maxcyte electroporation system. In our first two pre-GMP engineering runs, using plerixafor mobilized HSPCs, clinical scale CL1.1 cartridge, GMP-like Cas9 protein, and GMP-like sgRNA, we achieve ~92% editing in bulk HSPCs and further xenografted into *NBSGW* mice. At 17 weeks, we noticed 70-84% editing efficiency with no alterations to lineage or engraftment compared to unedited HSPCs. Furthermore, we observed 23.5-26% HbF in erythroid cells isolated from the bone marrow, well in range of expected therapeutic range. In summary, we describe a roadmap for rigorous evaluation of -115 γ-globin promoter editing approach to induce therapeutically effective levels of HbF for SCD therapy to support a planned SAGES1 first-in-human clinical trial.

311 Correction of Multisystem Smooth Muscle Dysfunction Syndrome in a Mouse Model via Base Editing

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Multisystemic smooth muscle dysfunction syndrome (MSMDS, OMIM #613834) is an ultrarare genetic smooth muscle myopathy with major dysfunction in the vascular, respiratory, enteric, and genitourinary systems. Cases described to date have been monogenic, associated with the missense variation at arginine 179 of the ACTA2 gene, most commonly creating a missense variant (R179H). Since this disease is caused by a single base G-to-A mutation, it is an ideal target for gene knockout or correction via CRISPR technologies. To scalably evaluate

effective genome editing approaches to correct the ACTA2R179H mutation, we first developed a clonal HEK 293T cell line bearing the R179H substitution. We designed and tested various combinations of prime editors and pegRNAs, selecting the most optimal pair to generate several ACTA2R179H cell lines of varying zygosity. Using heterozygous or homozygous cell lines, we evaluated dozens of CRISPR-Cas strategies to knockout or correct the mutant allele, via nucleases and base editors, respectively. With adenine base editors (ABEs), we achieved high levels of correction of the disease-causing G-to-A mutation, when paired with different gRNAs that position the edit window of the ABE deaminase domain over the mutated nucleotide (relying on either WT SpCas9 or our engineered PAM variants). Sequencing from transfections in the ACTA2R179H cell line revealed that although ABE8e-SpCas9 could correct the mutation, the intended edit was accompanied by very high levels of unwanted editing of nearby 'bystander' adenines. However, an SpCas9 PAM variant paired with an alternate gRNA (shifting the edit window of the base editor) enabled efficient and specific correction of the target base with minimal bystander editing. To investigate the translatability of our base editing approach in vivo, we created a knock in murine model of MSMDS with an exon advancement strategy that allows for controlled cre-inducible mutant allele expression (Acta2R179H/fl). The mutant allele in heterozygosity was activated using Cre recombinase driven by the smooth muscle cell (SMC) specific Myh11 promoter. Myh11-Cre:Acta2R179Hfl/+ mice exhibit multiple phenotypes consistent with MSMDS including neurovascular dysfunction with neurodegeneration, aortic enlargement, intestinal dysmotility, and premature death. For in vivo delivery of the ABE, we cloned an intein-split construct compatible with dual adeno-associated virus (AAV) delivery. The constructs were packaged in two AAV serotypes, including AAV9 and our novel AAV capsid optimized for murine vascular delivery. Analysis of Myh11-Cre:Acta2R179Hfl/+ mice retro-orbitally injected at P3 with dual AAVs revealed highly efficient and specific genomic correction of the R179H causing mutation in multiple tissues (~60% in hepatocytes). Importantly, the P3 injected mice exhibited a dramatic phenotypic recovery, including improved weight gain, rotarod and open field performance, reduced aortic diameters, and vastly extended life span. Together, we optimized an efficient therapeutic approach to improve clinical outcomes in a mouse model of MSMDS via in vivo base editing.

Pharmacology/Toxicology Studies: In Vitro and In Vivo Safety

312 Tissue-Restricted Promoter Selection as a Mitigation Strategy for Dorsal Root Ganglia Toxicity Due to AAV9 Treatment of Yucatan Pigs

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Adeno-associated virus (AAV)-induced dorsal root ganglia (DRG) toxicity has been observed in several species including nonhuman primates and pigs and is characterized by neuronal degeneration, mononuclear cell infiltration, and in rare instances, hindlimb ataxia and paresis (Hinderer et al. *HGT* 2018; Hordeaux et al. *HGT* 2020). Route of administration (ROA) and vector dose have been correlated with the severity of DRG toxicity, which can be mitigated by limiting transgene expression in DRG (Hordeaux et al. *STM* 2020; Guibinga et al. *ASGCT* 2022). We have developed a novel, endoscopic ultrasound (EUS)-guided, automated delivery system and route of administration (ROA) to enable direct injection of AAV to the pancreas to improve the risk-benefit profile of gene therapy for metabolic diseases, such as type 2 diabetes. Our aim was to characterize DRG toxicity due to AAV in our porcine preclinical model and develop a mitigation strategy for this potential off-target effect. Yucatan pigs (30-40kg) were utilized to establish the EUS procedure and pancreatic ROA since the porcine digestive tract anatomy, including the pancreas, is similar to humans. EUS-guided injection of AAV9 encoding for an enhanced green fluorescent protein (GFP) reporter under the control of a strong ubiquitous promoter (scAAV9-CMV-GFP) led to dose dependent pancreatic transduction, with greater than 40% of all cells exhibiting GFP expression at the highest dose tested. The dose range tested was 5E12-1.5E14 VG, corresponding to ~1E11-5E12 VG/kg, with a total of 11 pigs tested with scAAV9-CMV-GFP (n=2-4 per group). Biodistribution analyses from multiple organs are consistent with high pancreatic and limited off-target AAV tissue exposure. However, local pancreatic administration of AAV resulted in dose dependent AAV transduction of DRG. We also observed corresponding DRG toxicities including inflammation, elevated serum neurofilament light chain (NF-L), and hindlimb ataxia and paresis in one animal dosed at 5E13 VG (1.3E12 VG/kg). In order to assess promotor restriction as an additional DRG toxicity mitigation strategy, we tested 1E13-5E13 VG doses of an AAV encoding for GFP under the control of a β-cell-restricted promoter to prevent transgene expression in off-target tissues. Promotor restriction eliminated GFP expression in DRG and the spine as well as any signs of DRG toxicity, including inflammation or elevation in NF-L across all animals evaluated (n=7). This suggests that the initial DRG toxicity finding may have been due to the strength of the ubiquitous CMV promoter and/or the immunogenicity of the GFP payload as previously documented (Hordeaux et al. *HGT* 2020; Guibinga et al. *ASGCT* 2022). Together, these studies highlight the sensitivity of the pig as an animal model for DRG toxicity, as well as promoter restriction as a viable

strategy to mitigate DRG toxicity in addition to using a local ROA. Additional AAV, ROA, and procedure experiments and analyses are ongoing to evaluate the risk-benefit profile of local pancreatic gene therapy for metabolic disease.

313 Preclinical Safety Assessment of an Investigational Gene Replacement Therapy for the Treatment of *RPE65*-Mediated Inherited Retinal Dystrophies

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Background: Inherited retinal dystrophies (IRD) are a group of progressive blinding genetic diseases caused by mutations in any one of the over 250 causative genes. An estimated 1000-2000 people in the United States have the IRD disease caused by the mutations in *RPE65* gene, which encodes all-*trans* retinyl ester isomerase, an enzyme critical to the visual cycle. We developed an investigational gene therapy product, HG004, containing AAV-mediated human retinal pigment epithelium 65kDA protein (AAV-*hRPE65*) gene. Here we reported the results of its preclinical safety assessment study after injection in non-human primates (NHPs). **Methods:** HG004 was assessed in a 3-month good laboratory practice (GLP) toxicology study in NHPs after bilateral subretinal injection at three dose levels. Clinical observation, ophthalmoscopic examination (including optical coherence tomography, intraocular pressure, fundus photography, fundus fluorescein angiography, electroretinogram, and etc.), detection of vector shedding and immune response assessment were conducted during the study period. Histopathology and vector biodistribution in tissues were evaluated at 4-week and 13-week termination as well. **Results:** In terms of pharmacokinetic properties, HG004 was mainly distributed in the retina tissues, especially in the retina and choroid (above 10^6 copies/μg) (Fig.1a). The copy numbers of DNA and mRNA in other tissues were much lower than those seen in the retina tissues. No HG004 distribution was detected in the reproductive system on D92 (Fig.1a), and HG004 basically no longer entered the environment with the excretion 1-week post-injection (Fig.1b). Additionally, there were no drug-related systemic toxicity of HG004 observed after 4-week and 13-week post-injection. The adverse reactions (such as retinal injury at the site of needle entry, vitreous inflammatory cell exudation, and locally relevant changes in the area covered by the drug) mainly occurred in the retinas of both vehicle and tested group of monkeys, suggesting that these events were mainly caused by the injection procedure. Finally, the results of immunotoxicity assessment showed that there were no drug-related abnormalities found in whole blood samples of monkeys after injection, and no AAV or RPE65 antigen-specific T cells immune response was detected. **Conclusions:** Overall, HG004 was generally well tolerated, and there were no systemic toxicity reported. Therefore, HG004 is relatively safe for subretinal injection as a potential treatment of inherited retinal dystrophies due to *RPE65* dysfunction. The results of this China-manufactured gene therapy study for *RPE65*-associated inherited retinal dystrophies support the recent investigational new drug (IND) clearance from FDA in January 2023.

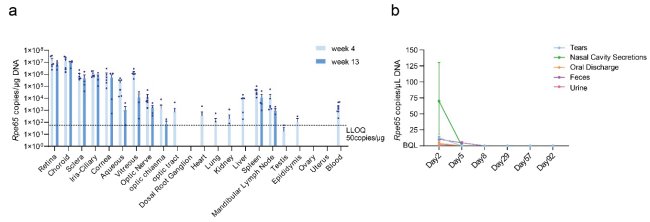

Figure 1. Biodistribution and vector shedding of HG004 in NHPs

314 *In Vitro* Toolbox to Investigate AAV-Induced Immune-Associated Hepatotoxicity

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Adeno-associated virus (AAV) vectors have emerged as promising in vivo gene delivery tools for gene therapy. They are mostly non-integrative, are able to transduce various tissues and have a low immunogenic profile. Despite these advantages, there are still reports of severe immune-associated hepatotoxicity after AAV administration in clinical trials. The mechanism behind this liver immune response is still not clearly understood. Here, we aimed to investigate the effect of AAVs on liver cells and to better understand which cell populations and by which mechanisms they may elicit a hepatic immune response. We used human liver in vitro models: HepaRG (hepatocyte cell line), hTERT-HSC (hepatic stellate cell line) and THP-1 (macrophage cell line, used as a Kupffer cell surrogate) that were exposed to different AAV-GFP serotypes (AAV2,3,8 & 9) and titers (MOI 10^3 - 10^6). Transduction efficiency was investigated by PCR, fluorescence microscopy and image quantification. Cellular responses were studied using cell viability and gene expression assays, immunofluorescent staining, and multiplex cytokine panel screens. Titer- and serotype-dependent decreases in cell viability were observed in HepaRG and hTERT-HSC but not in THP-1. Hepatic cells could be transduced using AAV, as the vector genome (GFP gene) could be detected in all three cell lines transduced with all four tested serotypes. However, protein expression of GFP was cell and serotype dependent. At a MOI of 10^6, AAV2 was able to transduce all three tested cell lines, whereas AAV3, 8 and 9 were able to transduce only HepaRG and THP-1. Transduction also elicited varied cellular responses: AAV2 increased αSMA gene and protein expression and decreased TGF-β gene expression in hTERT-HSC suggesting a direct activation of HSC. THP-1 appeared also activated by AAV2 and AAV8 as indicated by increased gene expression of CD80, CD206, HMOX and CXCL10 (IP-10). Immunostimulatory potential of AAV2 and AAV8 on hepatic cells was also suggested by the increased secretion of various pro-inflammatory cytokines and chemokines in all three cell lines, with IL-6 levels strongly increased in HepaRG and THP-1. Based on these studies in liver cell surrogates, an immune response to AAV-mediated liver gene therapy may be potentially driven by the activation of hepatic stellate cells and Kupffer cells, and increased secretion of

pro-inflammatory cytokines and chemokines by parenchymal and non-parenchymal cells. Further studies will focus on more complex 3D human in vitro models to gain insight into AAV-induced immune-associated hepatotoxicity and eventually help improving the safety of AAV vectors in gene therapy.

315 Investigating the Impact of Empty AAV Capsids on Safety and Efficacy Following Intracisterna Magna Administration in New Zealand White Rabbits

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Recent reports of treatment-emergent serious adverse events from recombinant AAV-mediated gene therapy have highlighted the need to better understand the effect of quality attributes of the AAV material on safety and efficacy outcomes. A particularly important topic which has been receiving a lot of attention is the impact of empty AAV capsids. Empty AAV capsids are process-related impurities generated during AAV production which lack the transgene DNA that is present in "full" AAV particles. There are significant gaps in understanding how the presence of empty capsids impacts safety and efficacy of AAV preparations. Additionally, there are no definitive regulatory guidelines on acceptable levels of empty capsids. Route of administration (ROA), dose, target, and indication can all potentially influence the impact of empty capsids, further increasing the complexity of this issue. To support an internal therapeutic program and using a rabbit model, we investigated the potential impact empty capsids may have on AAV performance *in vivo*. New Zealand white rabbits were dosed with 1.0E+13 genome copies of an AAV vector by intracisterna magna (ICM) injection. This AAV vector expresses a small RNA under the control of ubiquitous promoter and knocks down expression of human superoxide dismutase 1 (SOD1). Study materials were prepared by transient transfection in HEK293 cells followed by affinity chromatography purification. Cesium chloride (CsCl) gradient ultracentrifugation was then applied to isolate and enrich full and empty capsid populations. Target percentage full:empty ratios were achieved by combining these enriched populations and confirmed through analytical characterization. Rabbit study groups (n = 3 per group) were administered vehicle control, enriched full (1.0E+13 viral particles), 75% full and 25% empty (1.33E+13 viral particles), 50% full and 50% empty (2.0E+13 viral particles), and enriched empty capsids (1.0E+13 viral particles). The three groups which were administered full capsids all received the same genome copy dose. Findings were compared across groups to assess both transduction and safety endpoints. In-life observations, clinical chemistry, and hematology analyses showed no significant differences across study groups. Additionally, blood and cerebrospinal fluid (CSF) analysis of neurofilament (NF), a biomarker for neurotoxicity, showed elevations in study groups which received any percentage of full capsids, but no difference across these groups. Conversely, the vehicle control group and the group which received enriched empty capsids saw no elevations in NF. Biodistribution, transgene expression, and histopathology analyses are on-going. These results demonstrate that ICM co-injection of up to 1.0E+13 empty AAV capsid particles to rabbits does not significantly impact common safety endpoints. We will continue to evaluate the potential impact of empty capsids on transduction and microscopic pathology.

316 Intraparenchymal Dosing in Beagle Dogs Using Convection Enhanced Delivery Guided by Real Time MRI

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In the clinic, convection-enhanced delivery (CED) is routinely used in patients for targeted intraparenchymal brain dosing, spanning a multitude of neurological indications. As non-clinical toxicology studies require drug delivery methods to closely mirror those used in the clinic, expanding the number of non-rodent preclinical models for CED proves valuable. To achieve CED, standard procedures incorporate real-time MRI to target and verify local delivery to desired brain regions by employing a step-infusion paradigm coupled with a contrast agent such as gadolinium. We have established this procedure in non-human primates (NHPs) and have successfully delivered novel neurological therapeutics to multiple brain regions tailored to each respective indication. Expanding on this progress established in NHPs, we have developed a Beagle dog model as an additional non-rodent non-clinical alternative. Up to six different parenchymal sites per animal have been dosed at rates of 120 to 300 μL per hour with dose volumes ranging between 30 to 50 μL per site. The radial error in relation to the planned target is the submillimetric range. By incorporating real-time MRI, it is possible to accurately target relevant brain regions while also monitoring dose distribution over time. As observed in NHPs, Beagle dogs recover well from the surgeries and presented with minimal procedure-related clinical signs including mild tremors, cutaneous changes, and decreased activity which were expected after surgery and resolved within approximately 4-days after the procedure. From an ethical standpoint, Beagle dogs represent an advantageous alternative to non-human primates. This work demonstrates an additional non-clinical model for CED using real-time MRI, with a workflow that utilizes clinically relevant methods and devices, ultimately addressing the need for alternative non-rodent non-clinical models for assessing toxicology of neurological therapeutics.

317 Target Cell and Tissue Specificity of a Novel CD8-Targeted Fusosome for Direct *In Vivo* Delivery of CD19 or a CD20 CAR to CD8+ T Cells

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To overcome conventional *ex vivo* CAR T limitations, Sana is developing a novel gene therapy platform that can deliver CAR transgenes directly to T cells via systemic administration of a fusosome (a novel integrating viral vector whose target specificity can be altered by engineering viral envelope proteins). Although anti-CD19 CAR T cells have been clinically validated to be effective with an acceptable

safety profile, the use of a novel gene therapy approach for *in vivo* delivery rather than the conventional *ex-vivo* delivery presents unique safety challenges. To assess *in vivo* safety, we selected Nemestrina macaques as they are the only nonclinical model permissive to transduction by an integrating vector. Because the CD19-directed CAR does not cross react in macaques, we designed a surrogate vector with the same CD8-targeting fusogen, but with a payload to express a CD20-directed CAR, allowing us to assess: activity, biodistribution, and potential toxicities related to vector or CAR. To support the safety assessment, we evaluated the fusosome's CD8-targeting binder and CAR's on- and off-target binding by performing cell and tissue specificity: 1) testing a complete panel of frozen human and Nemestrina tissues in a tissue cross reactivity (TCR) study, and 2) evaluation of binding, expression, and activity in a select panel of primary and immortal cell lines as well as target cell co-culture systems. TCR: Representative scFvFc fusion proteins directed at either CD8, CD19 (human CD19+ tumor cell), or CD20 (NHP B Cell), were tested to evaluate cell and tissue specificity. The CD8 binder on our fusosome used to target CD8+ T cells is a single-chain variable fragment (scFv) that was isolated from a fully human synthetic scFv library via phage display. CARs are synthetic immune receptors (binders) that comprise an scFv that directs the pharmacological activity of CAR T cells to tumor target cells. Several rounds of screening against human CD8ab and cyno CD8a were performed to isolate binders with cross-reactivity to macaques. CD8+ SupT1 cells or CD19+/CD20+ Ramos cells served as assay positive controls for CD8 and B cell targets, respectively. TCR studies confirmed both cell and tissue specificity of all binders, with no detectable off-target binding of the CD8-directed fusosome, and specific binding of both CAR binders (CD19 or CD20) to B-cell rich regions of lymphoid tissues. In vitro: In primary cells, on-target binding was observed on T cells. Off-target binding and transduction of primary human hepatocytes was observed with the CD8 fusosome at very high concentrations, substantially above the intended clinical therapeutic range. Importantly, immunoreactivity of the CD8-directed binder to hepatic tissue was not noted in either human or macaque livers in the TCR study. Furthermore, an *in vivo* biodistribution study in Nemestrina, showed no evidence of hepatic transduction by *in situ* hybridization or quantitative ddPCR, suggesting minimal safety risk for the intended therapeutic range. Target cell specificity of CD19-directed and CD20-directed CARs was further confirmed in target cell killing assays that showed *in vitro* transduction of 1) human and NHP CD8+ and not CD4+ T cells, 2) target-directed killing of CD19+ NALM-6 tumor cells, 3) target directed killing of CD20+ K562 and not CD20- K562 cells when co-cultured. Taken together, the data confirmed specific CAR expression in human and NHP CD8+ effector T cells followed by specific CD19+ or CD20+ target cell engagement and cell killing, supporting evaluation of *in vivo* administration of this novel CD8-directed fusosome for CAR T therapies.

318 Safety and Biodistribution Study of Intra-Articular Delivered ICM-203, an AAV Gene Therapy Vector for Osteoarthritis, in Normal Rodents and Osteoarthritis-Induced Beagle Dogs

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Osteoarthritis (OA) is the most common degenerative joint disorder worldwide and a leading cause of locomotive disability in the elderly population. ICM Co., Ltd. has developed AAV based gene therapy for OA, called ICM-203. ICM-203 is an adenoviral-associated, recombinant gene therapy vector containing a bioengineered human Nkx3.2 transgene and intended for the treatment of patients with mild to moderate OA (KL grade 2 to 3). Nkx3.2 was initially identified as a pro-chondrogenic factor promoting cartilage development. Our previous studies have revealed that intra-articular (IA) delivery of ICM-203 was found to be capable of regenerating functional cartilage, suppress synovial inflammation, and restoring normal joint function in OA induced mice and beagle dogs. The IND-enabling safety and pharmacokinetic (PK) studies including the vector and mRNA biodistribution (BD) analyses were conducted in mice, rats, and beagle dogs. These study results were incorporated with toxicology studies and the results are presented here. PK studies were conducted in mice after ICM-203 IA injection. In the 26-week study ($1.4x10^{10}$ vg and $1.4x10^{11}$ vg per knee joint), vector DNA and transgene expression levels in target site remained relatively stable and persistent up to 26 weeks, demonstrating potentially prolonged therapeutic effects of ICM-203. Since ICM-203 is administered to a confined space of the joint; therefore, in the 90-day ($2x10^{10}$ vg and $2x10^{11}$ vg per knee joint) study, non-target distribution (blood, heart, kidney, liver, spleen, lung, lymph nodes, reproductive organs) of vector DNA and/or transgene expression was minimal. BD, toxicity, and immunogenicity of ICM-203 were examined in a GLP-compliant 90-day rat study, in which a single dose of ICM-203 was administered at $4x10^{10}$ to $4x10^{12}$ vg per knee joint via bilateral IA injection. ICM-203 vector DNA and transgene expression were quantitated with validated methods in the knee joint and non-target sites in the 90-day study course. Vector DNA and transgene expression levels were quantifiable in all tissues investigated at the high dose group with the highest vector DNA and transgene expression detected within the injection sites. No adverse effects were found in toxicological endpoints related to ICM-203 throughout the study. Formation of both neutralizing and transgene antibodies in response to the AAV5.2 capsid and expressed transgene protein, respectively, was confirmed and the IFN-γ splenocyte enzyme-linked immunospot (ELISpot) analysis showed immune recognition of the AAV5.2 capsid which was decreased over the 90-day time period. Twenty-seven-week safety study in OA-induced beagle dogs showed IA administration of ICM-203 with doses up to $2x10^{13}$ vg per knee joint was well tolerated. All general toxicological endpoints (mortality, physical examinations, gross pathology, hematology, clinical chemistry, and histopathological analyses) revealed no adverse effects related to ICM-203. The biodistribution analysis demonstrated that the majority of vector DNA and transgene expression retained in the injection site

for 27 weeks. Among non-target sites, very low levels (less than 1,000 copies of vector genome per microgram of gDNA) of vector DNA was detected in liver, spleen and lymph node. In addition, no abnormalities were found in terms of immunogenicity. Overall, these results were included in the ICM-203 IND submission and the program received no study hold comment from the US FDA in Oct 2022.

Cell Therapy Product Manufacturing

319 Efficient and Minimally Perturbative CAR-T Cell Engineering Using Peptide-Enabled CRISPR RNP Delivery

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Precision genome editing of primary human T cells offers the potential for sophisticated and durable cell therapies, but the use of electroporation for delivering genome-editing reagents is a source of cell toxicity and requires costly, cumbersome hardware and reagents. This manufacturing burden vastly limits the access and wide implementation of genome-edited T cell therapies. We developed a method called peptide-enabled RNP delivery for CRISPR engineering (PERC) to conduct precise editing without the need for electroporation. In this process, an RNP enzyme such as Cas9/gRNA or Cas12a/gRNA is mixed with a particular amphiphilic peptide and then applied to cells in culture, resulting in efficient gene knockout (up to 90%). Compared to electroporation, PERC produces significantly higher yields of edited cells, preserves the naïve and memory phenotype, and is less perturbative at the transcriptomic level. PERC can be paired with adeno-associated virus (AAV) bearing a homology-directed repair template for making efficient electroporation-free knock-ins, such as of a CAR transgene to the TCR alpha constant (*TRAC*) locus under endogenous promoter control, which has been shown to improve anti-tumor potency compared to virus-mediated pseudorandom integration. With PERC and AAV, we achieved up to 75% *TRAC*-CAR knock-in efficiency. As PERC is minimally perturbative, it can be used to edit multiple loci sequentially and thereby avoid chromosomal translocations, which arise when multiple RNPs are delivered simultaneously. Remarkably, we achieved above 25% dual knock-in at *TRAC* and *B2M* without detectable chromosomal rearrangement. Functionally, *TRAC* CAR-T cells produced using PERC maintained the advantage of their electroporation counterpart over pseudorandomly engineered CAR-T cells in targeting antigen-expressing tumor cells. Furthermore, multi-locus-edited CAR-T cells produced using PERC demonstrated therapeutic potency matching that of electroporation in an in vivo model of B-ALL. In summary, PERC paired with AAV enables efficient, convenient, non-toxic, minimally perturbative, and multi-locus editing. Looking forward, this approach has the potential to decrease the cell handling steps and infrastructure involved in making precisely engineered T cell therapies and could lower the barrier to point-of-care manufacturing.

320 A Minimal Workflow for *Ex Vivo* Magnetically-Assisted Lentiviral Vector Transduction of Hematopoietic Stem and Progenitor Cells for Gene Therapy Applications

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Currently, there are 8 approved lentiviral vector gene therapies (LV-GT) but none of these were developed in low-and-middle income countries (LMICs), which harbor 90% of the world's disease population (PMID:36203001). Viral vector remains a financial and temporal burden limiting availability to LV-GT in LMICs as well as high income countries. Here we tested a minimal workflow utilizing a LV with high transduction efficiency at low doses to circumvent this limitation. Primary human hematopoietic stem and progenitor cells (HSPCs, CD34+) from G-CSF mobilized adult donors were transduced with either cocal virus envelope glycoprotein pseudotyped LV (cocal LV) or vesicular stomatitis virus envelope glycoprotein pseudotyped LV (VSV-G LV) encoding a green fluorescent protein (GFP) transgene. We evaluated three transduction processes: standard culture-based transduction, spinoculation and magnetically-assisted transduction (MAT). HSPC were pre-stimulated for 16h in StemSpan™ SFEM II media containing recombinant human stem cell factor (SCF), thrombopoietin (TPO) and Fms-like tyrosine kinase 3 ligand (FLT3-L) at 100ng/mL each. Cells were either first spinoculated at 400g for 1h or directly cultured in the presence of LV at 37°C, 5% CO_2. For MAT, LV were incubated in ViroMag STEM™ at a dilution of 1:500 for 20 minutes, then added to HSPC in culture media and placed on a CTS™ DynaMag™ magnet for 20 minutes. In all experiments cells were seeded at 2 x10^6/mL, transduced once at 10 transducing units (TU) per cell and cultured post-transduction in Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum and SCF, TPO, and FLT3-L for 10 days. No difference in viability or expression levels of low-density lipoprotein receptor (LDLR), the described receptor for both VSV-G and cocal, was observed across any condition. GFP detected in cells gene modified by cocal LV was 10-60% higher than in cells modified by VSV-G LV. MAT yielded 5-fold higher transduction efficiency in CD34+ cells in comparison to standard culture-based transduction and spinoculation. We evaluated *in vivo* engraftment of MAT-treated cells in *NOD/SCIDgamma*$^{-/-}$ (NSG) immunodeficient juvenile mice. Each mouse was injected with 1x10^6 human HSPCs either freshly transduced or transduced and then cultured in StemSpan™ for 2 days. Mice transplanted with freshly transduced cells had higher levels of engraftment (hCD45+) without transduction effects compared to mice receiving cultured cells ($p<0.005$). *In vivo* gene marking of total PB leukocytes at 28 weeks of age will be assessed at end of study. These

data suggest that MAT of cocal LV at low doses can greatly reduce viral vector burden for LV-GT. These data demonstrate proof of concept for a cost-effective minimal workflow for manufacturing LV-GT.

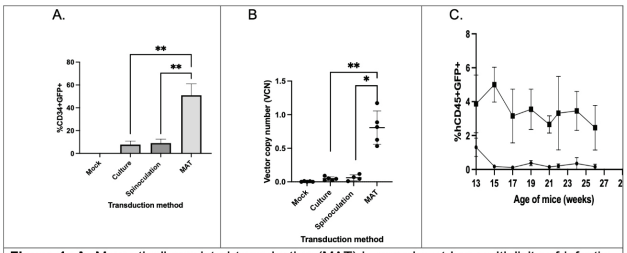

Figure 1: A. Magnetically-assisted transduction (MAT) is superior at low multiplicity of infection (MOI). Human CD34+ cells transduced with cocal LV at a single MOI of 10 TU/cell. **B.** Gene modified CD34+ cells had low LV copies per genome hence safe to use. **C.** Cultured CD34+ cells had better transduction (GFP+) than fresh cells.

321 'Off the Shelf' CD62L-Selected Multiplex Edited Umbilical Cord Blood CAR T Cells

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Most allogeneic CAR T cell applications modify healthy donor lymphocytes, derived from adults under steady state apheresis conditions. Umbilical cord blood (UCB) T cells represent an attractive source of naïve T cells given their distinct ontogenetic origins and may offer enhanced properties of expansion and activation, compared to adult peripheral blood lymphocytes (PBL). By exploiting a characteristic feature of UCB T cell naivety, the expression of CD62L, T cells were enriched at scale using magnetic bead conjugated antibodies ahead of a semi-automated and compliant process. Selected CD62L+ UCB T cells were readily amenable to transduction with a CRISPR-CAR19 lentiviral vector, encoding CRISPR cassettes within the long-terminal repeats (LTRs), which mediated multiplexed editing of TCR α chain and CD52 gene after electroporation of SpCas9 mRNA, yielding cells with negligible GvHD potential and resistant to serotherapy, respectively. The resulting 'universal' CAR T cells were highly enriched for CAR expression (~85%) with residual TCRαβ T cells <1% in the final product. High-throughput molecular investigations confirmed on-target fidelity of genome edits with no significant 'off-target' editing at Digenome-Seq informed sites of predicted guide dependent activity. Predictable chromosomal translocations were quantified by digital droplet (ddPCR) and Fluorescence in situ Hybridization (FISH) (≤1 %) and other aberrations excluded by karyotype analysis of metaphase spreads. UCB CAR19 T cells mediated potent cytolytic activity against CD19+ Daudi targets and appropriate secretion of cytokines IFN-γ, TNF-α, IL-4 and IL-2 in vitro and demonstrated robust anti-leukemic effects in vivo using a xenograft model of B-cell malignancy, at levels comparable to PBL-derived CAR T cells. UCB enriched for CD62L-positive cells may provide an alternative source for banking universal CAR T cells from donors with common HLA types, which could reduce the need for intense lymphodepletion currently required for allogeneic CAR T cells.

322 Live Imaging on Single Cell Arrays (LISCA) as a Platform to Study Dynamics of mRNA Expression in Mammalian Cells

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Introduction: mRNA based therapies evolved into one of the most powerful therapeutic technologies. Over the last decades much effort was put into enhancing mRNA delivery and translation efficiency. The advent of lipid nanoparticles (LNPs) brought RNA delivery and in-vivo efficacy to the level of clinical applications. However, at the single cell level, delivery of LNPs is heterogeneous and the expression level and timing is poorly controlled. As opposed to the need of some applications local overexpression might have detrimental effects. In an effort to gain more control over expression, we utilize a frequently occurring motif in natural gene regulation employing downregulation via siRNAs. So-called incoherent feedforward loops (icFFLs), consisting of initiation and simultaneous or delayed down-regulation, lead to faster response of the out-put signal with reduced noise. Here we realize a regulatory gene expression motif encoded in lipid nanoparticles containing eGFP mRNA and eGFP targeting siRNA (Fig. 1). We show that codelivery of these counteracting components represent a icFFL with the expression output being determined by the ratio of mRNA and siRNA.

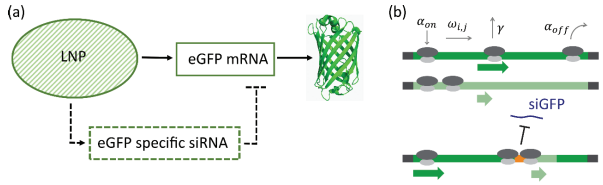

Figure 1: Controlling mRNA delivery: (a) icFFL LNP model and (b) modulation of ribosome movement to control mRNA stability under RNAi attack

Secondly, we demonstrate control over mRNA stability via codon modification. Synonymous re-coding of the mRNA's open reading frame is one approach to investigate and optimize the physics of mRNA stability. We evaluate the potential of bias in codon usage on influencing the ribosome density on the ORF and therefore the mRNA's translation and degradation kinetics.
Methods: We use live imaging on single cell arrays (LISCA) to record eGFP expression kinetics after transfection of hundreds of single cells in parallel. The approach enables high throughput investigation into transfection homogeneity to study the effect of icFFL LNPs. Describing the translation in biochemical rate equations, we analyse mRNA expression rates and stability at single cell level (Fig. 2).

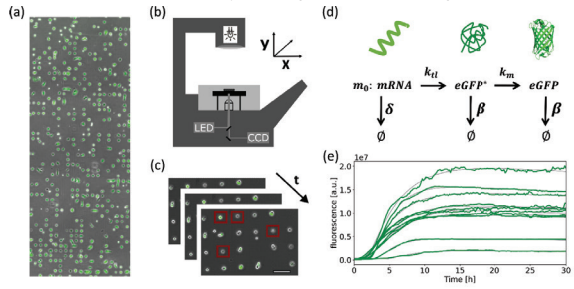

Figure 2: LISCA platform: (a) Exemplary overview of single transfected cells, (b) Time-lapse microscope setup (c) Time resolved images are analysed with in-house software (d) three stage maturation model to fit trajectories (d) exemplary fluorescence trajectories (green) with fit (grey)

Results: We find faster and more homogenous expression in eGFP time courses in cells transfected with icFFL LNPs. The asymptotic expression levels show power law decrease as a function of siRNA/mRNA ratio. Our approach demonstrates self-regulated expression via icFFL LNP based genetic programs. Furthermore, we could show that insertion of slow codon windows in the ORF enables tuning of the mRNAs stability under siRNA attack. We observe distinct differences in degradation rates for GFP mRNAs with various ORFs with different ribosome jam provoking slow codon windows in agreement with simulation. This change in stability is dependent on the absence or presence of siRNA.

323 Development of a GMP-Compatible Manufacturing Process for Highly-Edited, Stem-Like, Metabolically Fit, Virus Free CRISPR CAR T Cells

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Six Chimeric Antigen Receptor (CAR) T-cell therapies have been FDA-approved to treat liquid tumors spawning immunotherapy research for a wide range of cancer indications. However, limited clinical success of these products in solid tumors due to a lack of persistence and anergic T-cell phenotypes have led researchers to search for new ways to manufacture CAR T cells. Recently, virus-free CRISPR CAR T cells have been produced through CRIPSR/Cas9-induced knockout of the endogenous TRAC gene. There cells have a favorable memory-enriched phenotype and can be manufactured quickly. Here, we further improved critical quality attributes of VFC CAR T cells through the addition of small molecules and a novel media switch post-electroporation (EP) to produce highly-edited, 'metabolically fit' cells. Cells were evaluated using spectral cytometry, Seahorse assays, and within in vivo xenograft neuroblastoma mouse models. Highly-edited VFC CAR T cells, up to 50% transgene positive, can be produced with the addition of the DNA-PK inhibitor M3814 (Nedisertib) post-EP. Additionally, the usage of two different basal medias, TexMacs and Immunocult XF, pre and post-EP respectively, endows VFC CAR T cells with superior efficacy in in vivo mouse models and a 'metabolically fit' phenotype that favors oxidative phosphorylation. This 'metabolically fit' phenotype combines glutamine and glucose restriction pre-EP which allows the cells to favor oxidative phosphorylation over glycolysis, to prime the cells for persistence in a hostile tumor environment and favors the formation of long-lasting memory cells. These findings shed light on the ease and efficacy of simple media changes during manufacturing to produce memory-enriched, potent T cell therapies.

324 Improving Genome Editing Efficiency and *In Vivo* Potency of CAR T Cells via Label Free Metabolic Imaging

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Introduction: While Chimeric Antigen Receptor (CAR) T therapy is a promising treatment for hematological malignancies, its translation to solid tumor remains challenging. The number of CAR$^+$ cells (CAR T dosage) and stem-memory cell phenotypes have been correlated with long term treatment response. Low CAR knock-in efficiency can be compensated by long *in vitro* expansion to reach the desired dosage, but this causes T cells differentiation and exhaustion. Hence, strategies to improve nucleofection efficiency and expansion condition for a potent, persistent CAR T product is critical. As T cell metabolism strongly correlates with function, we used Optical Metabolic Imaging (OMI) to identify the optimal nucleofection timeframe to achieve highest genome editing efficiency, and the media-cytokine condition for highest *in vivo* CAR T efficacy. OMI allows non-invasive, real-time characterization of cell metabolism based on autofluorescence from metabolic coenzymes NAD(P)H and FAD by quantifying their relative abundance and binding activity.
Methods: Primary T cells were isolated from three healthy donors and activated with StemCell (αCD2/αCD3/αCD28) or TransAct antibody (αCD3/αCD28) for 12-72 hours prior to nucleofection for GD-2 CAR expression. T cell metabolic profile at the nucleofection timepoint was characterized with OMI. GD-2 CAR T cells were then expanded in a combination of media (ImmunoCult vs TexMacs) and cytokine (IL-2 vs IL-7) for 5 days before being evaluated for %CAR positivity, CAR T cell phenotypes, and OMI metabolic features. 5 million CAR$^+$ T cells were injected into NSG mice carrying xenograft GD-2$^+$ CHLA-20 and treatment response was determined after 21 days.

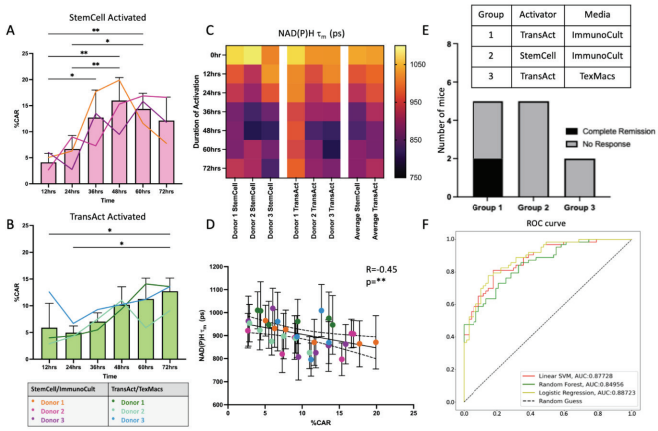

Figure 1. OMI identified optimal nucleofection timeframe and expansion condition for highly potent CAR T cells. (A-D) StemCell and TransAct activated T cells yielded highest %CAR positivity and lowest NAD(P)H t_m with activation duration of 36-48 hours and 48-72 hours, respectively. (E-F) Group 1 CAR T cells achieved highest rate of *in vivo* remission. OMI features allowed classification of CAR T cells from group 1 versus others with high accuracy (area under ROC curves > 0.80). *p<0.05, **p<0.01.
Results and Conclusions: T cells with different activation duration

and strategies yielded CAR knock-in efficiency varying from 5-20%, with StemCell and TransAct activated T cells having highest editing efficiency at 48 hours and 48-72 hours post activation, respectively (Fig. 1A-B). OMI revealed significant metabolic changes in T cells over the duration of activation, with lowest NAD(P)H mean lifetime (NAD(P)H t_m) observed in T cells activated for 36-48 hours with StemCell antibody and 48-72 hours with TransAct antibody, aligning with the timepoints of optimal transfection efficiency (Fig. 1C). OMI metabolic features (specifically NAD(P)H t_m) at nucleofection showed a significant negative correlation with CAR knock-in efficiency (Fig. 1D). CAR T cells with different activation and expansion conditions showed varied *in vivo* potency with the same dosage (Fig. 1E). CAR T cells from group 1 (TransAct activated, ImmunoCult + IL-2 expanded T cells) achieved highest rate of *in vivo* remission and displayed distinct metabolic features. A logistic regression classifier was successfully trained to classify highly potent CAR T cells (group 1) versus others based on OMI measurements with >80% accuracy (Fig. 1F). Our data demonstrated that OMI is sensitive to inform the optimal gene transfer timeframe and expansion condition to achieve potent CAR T cell products.

325 Evolution of a Hyperactive TcBuster Transposase Enables Highly Efficient Non-Viral Generation of CAR-NK and CAR-T Cells for Combination Cell Therapies

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Immunotherapy with chimeric antigen receptor (CAR) expressing NK and T cells engineered through viral vectors has shown exceptional efficacy against hematologic cancers in clinical trials. This method, however, is limited in cargo capacity, carries the risk of insertional mutagenesis, and large-scale manufacturing for clinical use is cost-prohibitive. CAR delivery via DNA transposon engineering is a viable alternative due to expedient and cost-effective production when compared to viral-based approaches. Here, we evolved and deployed a hyperactive TcBuster™ transposase (TcB-M) to deliver a CD19-CAR-DHFR-EGFP expression cassette (3.7 kb) to both primary human peripheral blood NK and T cells to achieve >90% CAR+ integration. Previously, the use of transposons in primary immune cells has been hindered by DNA toxicity and induction of a type I interferon response. We optimized methods to avoid this, including using DNase in recovery media and transposon delivery via Nanoplasmid vectors which have a small (<500 bp) backbone, high supercoiling, and can be rapidly moved towards GMP compliance. Our cargo contained a mutant dihydrofolate reductase (DHFR), resulting in our ability to select for CAR NK and CAR T with stable transposon integration using methotrexate (MTX) during manufacturing (**Fig 1A**).In vitro, CAR NK and CAR T cells elicited potent cytotoxicity against CD19+ Raji target cells within 24 hours (**Fig. 1B & C**). Within an in vivo Raji-challenged xenograft model, we found significant improvements in survival and decreases in tumor burden with single-dose therapies generated using TcBuster. To avoid CRS associated with high-dose CAR T therapy, we demonstrated that a sub-optimal dose of CAR T can be combined with CAR NK cells to improve survival with a single dose (**Fig. 1D**).

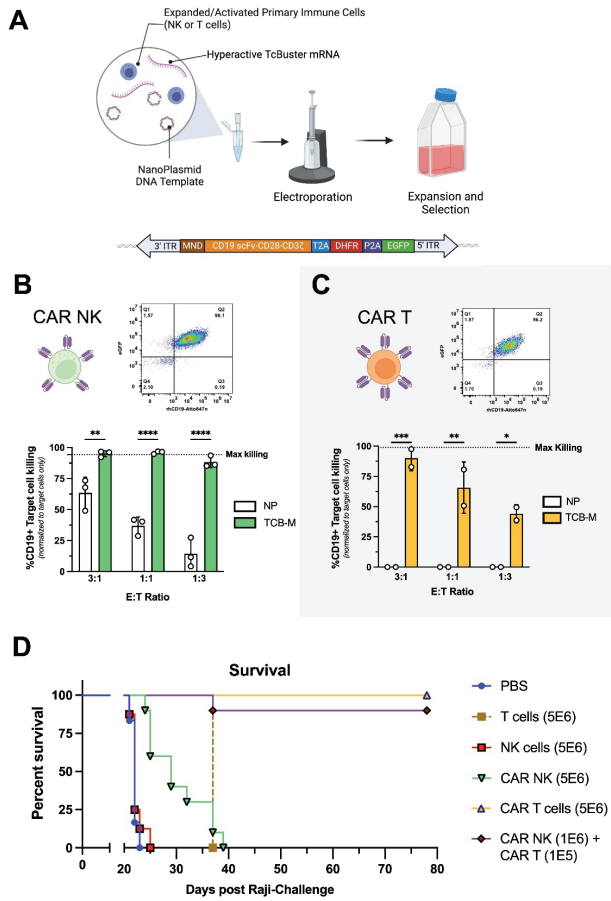

Figure 1. CAR NK and T cells engineered via TcB-M show enhanced in vitro and in vivo specific killing of target Raji cells and can be combined for an effective single-dose combination therapy. (A) Summary of hyperactive TcBuster (TcB-M)-based primary immune cell engineering with large-cargo construct (3.7kb, bottom) and expansion. (B) MTX-selected NK cells (n=3 donors) or (C) MTX-selected T cells (n=2 donors) from the pipeline show near 100% CD19-CAR expression and were co-cultured with luciferase-expressing, CD19+ Raji cells. Specific killing was measured by a luciferase-based assay. (D) Raji-tumor bearing NSG mice treated with a single dose CAR NK, CAR T, or a combination of sub-optimal CAR T + CAR NK show improved survival and control of tumor growth. Our work reveals an enhanced platform for rapid delivery of multicistronic vectors via transposition in both primary human NK and T cells. Through combining CAR NK and sub-therapeutic CAR T dosages, we were able to show significant improvements in survival outcomes in a xenograft tumor model that may translate to improved clinical outcomes with reduced risks of CRS development. Overall, this

non-viral approach to engineering represents a versatile, rapid, safe, and cost-effective option for manufacturing of cellular therapies over traditional viral delivery methods.

AAV Development for Eye, Muscle, Kidney and CNS

327 Massive Diversity Capsid Screening and Machine Learning Identify Next-Generation AAV for Targeted Tissue Biodistribution

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Gene therapies using adeno-associated viruses (AAVs) hold great promise for the treatment of a wide range of diseases. However, current therapies using wild-type AAV often suffer from high transduction of off-target tissues, particularly in the liver and dorsal root ganglia (DRG). This limitation decreases therapeutic efficacy and increases toxicity, making the development of improved AAV capsids essential to advancing the clinical application of gene therapy. Previous capsid engineering strategies have generally been limited to libraries of relatively low diversity (e.g., <1 million capsid variants), thus sampling only a small portion of the vast possible space of capsid design, and typically only comparing single target tissues of interest to liver. To overcome these limitations, the AAVid™ capsid discovery platform leverages massive diversity capsid libraries and direct-to-non-human primate (NHP) biological selection to generate a high-resolution map of how the AAV mutational space affects capsid assembly and tissue tropism. A combinatorial library of one billion capsid variants was generated, featuring mutations in the sialic acid binding pocket of the AAV5 backbone (AAV5id). This library was screened in four adult cynomolgus macaques (two males, two females), resulting in the recovery of over 30 million unique AAVid capsid sequences from over 50 distinct tissues. A novel computational pipeline was developed to analyze this primary dataset, allowing for the identification and advancement into functional secondary screens of high-confidence capsid variants for a given tissue of interest (positive selection) while de-targeting other tissues (negative selection). Additionally, we developed generative machine learning (ML) strategies to design novel, unseen *in silico* capsid variant sequences predicted to have a high probability of tissue specificity. Using these methodologies, we identified CNS-, skeletal muscle-, and cardiac muscle-targeting capsid variants following intravenous administration of the primary screening library in NHPs. We validated these capsid variants in a secondary screen in four additional NHPs that quantified targeted functional transduction using universal or cell type-specific promoters. From this screen, we identified capsid variants with 10x to 1000x enrichment of DNA and RNA in target tissues relative to all other harvested tissues and compared with wild-type AAVs. Notably, our unseen, ML-designed capsid variants were overrepresented among the tissue-specific variants, highlighting the power of our generative ML approach. Our liver inclusion and exclusion score achieved an AUC of 0.96, allowing us to a priori design capsids with greatly reduced risk of hepatotoxicity. Overall, our results demonstrate the success of ML-guided discovery of next-generation AAV capsids with targeted biodistributions. With our data spanning nearly 50 unique tissues in NHP, this new generation of tissue-tropic AAVs will help advance the development of more effective and safer gene therapies against a wide variety of diseases.

328 Side-by-Side Comparison of Systemic AAV8, 9 and rh74 Transduction in Human Muscle

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Adeno-associated virus (AAV) serotype-8, 9, and rh74 are currently used in multiple clinical trials to treat inherited neuromuscular diseases (e.g., Duchenne muscular dystrophy, spinal muscular atrophy, and myotubular myopathy, etc.). These serotypes were chosen mainly because of their outstanding muscle transduction efficiency in mice following intravenous injection. It is unclear whether this superior systemic muscle transduction can be translated to humans. Previous studies have shown that AAV performance is species-dependent (Human Gene Therapy 24:584-594, 2013; Molecular Therapy-Methods & Clinical Development 1:14002, 2014; Human Gene Therapy 26:54-61, 2015). There is an urgent need to profile AAV transduction in human muscles following systemic delivery. To address this issue, we developed a human muscle xenograft model. In this model, human donor muscle was engrafted in the tibialis anterior compartment of adult immune-deficient NSG mice. Human muscle regeneration was examined 1, 2, and 4 months after grafting. Human muscle underwent degeneration and necrosis in the first month. Robust regeneration was observed in the second month by embryonic myosin heavy chain immunostaining. Human muscle was fully regenerated by the 4th month and expressed both type I and type II myofibers. On morphometric quantification, human myofibers occupied >75% of the total area of the grafts. A pilot dose-finding study was conducted using an AAV9 vector that expressed the heat-resistant placental alkaline phosphatase reporter gene under the transcriptional regulation of the Rous sarcoma virus promoter (RSV.AP). The dose of 5E+12 vg/kg yielded unsaturated AP expression and was used in subsequent studies. AAV8.RSV.AP (n=6), AAV9. RSV.AP (n=4), and AAVrh74.RSV.AP (n=5) were delivered to mature human muscle grafts via tail vein injection. One month after AAV injection, we quantified (i) AP expression by histochemical staining in cross-sections, (ii) AP activity in tissue lysate, (iii) AAV genome copy number, and (iv) AAV transcript copy number in human muscle grafts and host mouse livers. We have collected tissues from injected mice and are in the process of quantifying DNA, RNA, and protein-level transduction. We will report our results at the annual conference.

329 Identification of Natural Retinotropic AAV2 Variants with Functionally Enhanced VP1 Unique Regions

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Adeno-associated viruses (AAVs) have become the ideal gene therapy vectors, particularly for treating inherited retinal diseases (IRDs). Several AAV vector-based clinical trials for IRDs are currently underway. Unfortunately, the large diversity of IRDs, which affect multiple retinal layers and cell types, complicates the development of novel treatments.Furthermore, concerns for low transduction profiles, vector-mediated toxicity at high doses, and immune side effects have sparked the need for novel capsids that can outperform contemporary serotypes following intravitreal (IVT) injection. To meet this need, we have identified from human tissues >200 AAV proviral capsid sequences and have screened their capacity to transduce retinal tissues by IVT injection in mice. Through this approach, we have identified two AAV serotype 2 (AAV2) variants that confer strong tropism to the retina. Interestingly, we found that these variants only differ from AAV2 by one or two residues found in the VP1 unique (VP1u) region. This poorly-studied region contains an essential phospholipase A2 (PLA2) domain, a calcium-binding domain, and nuclear localization sequences. VP1u is reported to play crucial roles in endosomal trafficking and escape, nuclear entry, and genome release *vitro*. Recent research by others have shown that enhancing PLA2 activity can simultaneously improve transduction and reduce immunogenicity, but how the PLA2 domain participates in AAV transduction and affects tropism remains unclear. We found that the two VP1u-altered variants, tentatively named v149 and v152, showed three-fold better transduction than AAV2. Furthermore, v149 and v152 exhibited near equal levels of transduction *in vitro* and *in vivo* as the leading retinotopic capsid, AAV2.7m8. When scAAV vectors packaged with v149 and v152 capsids are delivered by IVT injection in mice, transduction of amacrine and horizontal cells are two-fold better than what is achieved by AAV2 and AAV2.7m8; and two-fold more in ganglion cells and three-fold more in photoreceptors than conferred by AAV2. In exploring the function of the variant-defining residues, we showed that they confer higher cell surface binding and faster perinuclear localization. We also combined the variant-defining residues into one novel capsid design (AAV2.MC1), and tested its performance *in vivo*. We found that AAV2.MC1 is four-fold better than AAV2.7m8 following IVT injection (1E9 vg/eye). Furthermore, we demonstrate that because the VP1u region is highly conserved between the known AAV serotypes, the variant-defining residues of v149 and v152 have the capacity to be grafted onto other engineered capsids to further boost performance. For example, grafting the VP1u variant residues onto AAV2.7m8 increased transduction efficiencies in the mouse retina by three- to five-fold. Our major finding from this study is that transduction can be significantly impacted by the VP1u region, a domain that remains relatively untouched from current AAV capsid engineering efforts. These insights uncover new potential strategies for enhancing AAV vector performance via improving mechanisms for post-entry. This work also provides new retinal gene therapy capsids for evaluation.

330 Modification of Capsid Surface Residues in AAV2 for Improved Retinal Tropism

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Recombinant adeno-associated virus (AAV) vectors are the current gold standard in retinal gene therapy. The availability of several naturally occurring serotypes combined with low toxicity and well-established production methods favor their use as vectors for gene therapies. In the context of retinal diseases, the AAV2 serotype is the most commonly used vector with a good native tropism for retinal cells. Despite the success, there are still hurdles, such as the need of challenging injection routes and viral component-induced immune responses after vector administration. To overcome such obstacles, AAVs with improved characteristics are needed. Here we aimed to optimize previously described next generation AAV variants by rational design to optimize cell-specific targeting and/or mitigate immune responses. Specifically, starting with AAV2.GL and AAV2.NN, surface exposed residues known to be part of previously described antibody recognition sites were modified by either peptide insertion or amino acid substitution, or a combination of both. The resulting variants, namely AAV2.GL.F, AAV2.GL.G, AAV2.GL.453 and AAV2.NN.F, AAV2.NN.G, AAV2.NN.453 could be produced at stable titers and lead to high transduction efficiencies *in vitro* (e.g. 66.19%, 53.06% 72.08%, compared to parent variants with 13.75% and 35.99% at a MOI of 250). Escape from neutralization was assessed using an *in vitro* neutralization assay. After pre-incubating the novel variants with human serum containing neutralizing antibodies, the transduction properties of the vector/serum mixture were evaluated. AAV2.GL and AAV2.NN already showed lower neutralization than wild type AAV2. Despite their improved transduction efficiency, the new, further modified capsid variants could not reduce neutralization by various human sera tested beyond that observed for AAV2.GL and AAV2.NN. The promising transduction potential was further examined after intravitreal injection in wild type mice by *in vivo* confocal scanning laser ophthalmology and by immunohistochemistry. To examine the superior behavior of the new capsid variants in a dose dependent manner, two doses (1E09 and 5E09 total vg) were tested. Results showed superior transgene expression three weeks post injection in all novel capsid variants compared to AAV2.GL and AAV2.NN. At the low dose, AAV2.GL.F was the variant that produced the highest retinal gene expression *in vivo*. The superior transduction behavior was also confirmed in an *ex vivo* human retinal tissue culture model, where all novel capsid variants resulted in high transgene expression. These results suggest that modification of individual capsid surface residues allows further enhancement of the transduction properties, efficiency, and tissue tropism of AAV capsids, while it may not be sufficient to attenuate neutralization.

331 Evolving Nephrotropic AAV Variants Using Ex Vivo NHP Kidney Perfusion and Human Kidney Organoids

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Kidney diseases are estimated to affect 37 million people in the US, with nearly 810K Americans living with kidney failure - underscoring the crucial need for effective kidney gene delivery vehicles. Building on previously reported success in engineering cross-species compatible AAV (ccAAV) capsids, we adapted our structure-guided approach to evolve new variants in human kidney organoids. In addition, we developed an ex vivo rhesus macaque kidney perfusion using both ureteral and arterial routes to cycle AAV capsid libraries. By employing evolution schemes across different kidney systems (intravenous in mice and pigs, human organoid cultures and ex vivo organ perfusion), we discovered multiple new AAV variants. Notably, the ureteral and arterial perfusions yielded distinct capsid sequences, highlighting the importance of route of vector administration. High capsid protein sequence convergence was also observed in the case of several variants enriched from different approaches. Two novel AAV variants, AAV.k20 and AAV.k13, displayed robust transduction efficiency in murine kidneys following IV administration. Both variants revealed higher transgene expression compared to AAV9 and AAV.cc47, in particular, within the proximal tubules as indicated by immune-colocalization with lotus tetragonolobus lectin (LTL) staining. We also observed robust transgene expression using AAV.k20 and AAV.k13 vectors in human kidney organoids, corroborating murine data. The approach described here can also potentially be extended to ex vivo human kidney perfusion. Nephrotropic AAV variants represent promising delivery vehicles for therapeutic kidney gene transfer applications.

332 An Integrin-Targeting AAV Developed by a Novel Computational Rational Design Methodology Presents an Improved Targeting to the Skeletal Muscle and Reduced Tropism to the Liver

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Adeno-associated virus (AAV) have shown potential as a gene therapy vector due to its efficiency in delivering transgenes and low toxicity. However, its effectiveness can be limited by non-optimal tissue targeting and accumulation in the liver. High doses are often required to achieve therapeutic efficacy, particularly in the treatment of muscular diseases where the target tissue represents half the body mass. Such doses can lead to severe adverse effects, including hepatotoxicity as seen in recent clinical trials. With primary aim to lower treatment doses, we present here a novel methodology for AAV computational rational design targeting skeletal muscle, resulting in a new myotropic AAV variant. First, integrin alpha V beta 6 (AVB6) was selected from screening of potential receptors with high enrichment in skeletal muscle tissue compared to other organs. A liver-detargeting AAV capsid backbone, a hybrid between AAV-9 and -rh74 (AAV9rh74), was then engineered to specifically bind to AVB6. Inspired by recent successes of various protein design studies, we modified the entire loop of variable region IV from its predicted 3D structure in such way that the designed capsids acquire the RGDLxxL/I motif structure, which allows a high-affinity binding to AVB6 but remain stable thanks to low estimated energy. Five AAV variants from *in silico* design were experimentally tested. Compared to AAV9 or AAV9rh74, all designed AAVs showed similar productivity, better binding to AVB6, and greater infectivity towards human differentiated myotubes and murine skeletal muscles *in vivo* while entering poorly in the liver. One notable variant, LICA1, greatly increases transgene expression at 16.6/25.0-fold greater in human myotubes, and murine skeletal muscle at 13.7/129.3-fold greater than AAV9 and AAV9rh74, respectively. We further examined its efficacy, in comparison with AAV9, in delivering therapeutic transgenes in mouse models of Duchenne muscular dystrophy and limb-girdle muscular dystrophy R3 at a low dose of 5E12 vg/kg. At this dose, AAV9 is suboptimal where it can only infect 22.1-58.1% of total muscle fibers. In contrast, LICA1 effectively delivered and expressed transgenes in 74.8% myofibers in severely affected diaphragm, or at almost 100% in other tested muscles. Consequently, LICA1 corrected pathohistology by lowering centro-nucleation index and fibrosis, and restored global transcriptomic dysregulation. Interestingly, the anti-capsid antibody in LICA1 one-month treatment in DMD mice halves compared to AAV9 indicating lower immunogenic profile. Furthermore, compared to state-of-the-art myotropic AAVs identified by high-throughput screening, LICA1 is more specific since it targets skeletal muscle at similar level but accumulates less in liver. These results suggest the potential of our methodology for AAV engineering and our designed AAV for the gene therapy treatment of muscular diseases. The manuscript of this work is under preparation for submission.

Genome & Epigenome Editing Technologies II

333 A Novel Engineered U7 Small Nuclear RNA (snRNA) Scaffold Increases ADAR-Mediated Programmable RNA Base Editing

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RNA base editing presents a promising therapeutic approach for treating genetic disease, alleviating concerns of permanent DNA damage or immunogenicity from foreign bacterial proteins such as CRISPR/Cas. The naturally expressed human Adenosine Deaminase Acting on RNA (ADAR) enzyme can be directed to edit specific RNA targets by antisense guide RNAs (gRNAs), which anneal to a transcript and recruit ADAR to create targeted adenosine-to-inosine (effectively A>G) changes. For durable and potential one-time therapies, gRNAs can be delivered in genetically encoded form. However, a major challenge for RNA editing with gene-encoded gRNAs and endogenous levels of ADAR is achieving sufficient expression and function of the gRNA inside target cells, especially in

therapeutic situations where AAV delivery may be limited to around one viral genome per cell. Here we show that embedding antisense gRNAs into the framework of a natural small nuclear RNA (snRNA) design greatly increases the efficiency of custom RNA editing. We demonstrate that ADAR gRNAs coupled to an Optimal Sm-binding domain (SmOPT) and U7 snRNA hairpin sequence increased RNA base editing relative to ADAR gRNAs without the snRNA framework. Furthermore, robust editing was observed without the need for ADAR overexpression, demonstrating the recruitment of the cells' endogenous ADAR for programmable RNA editing. Adding an hnRNP A1 domain to the antisense guide RNA further improved editing. Importantly, no unwanted effects on transcript splicing or knockdown were detected. The increased editing efficiency provided by the SmOPT and U7 hairpin allowed for detectable RNA editing from a single genomically-integrated copy of gRNA construct per cell, which enabled us to further evolve the system by performing a pooled library screen of >700 gRNA variations against three separate gene transcripts in cell culture. This approach allowed us to identify critical residues required for RNA editing and revealed improved SmOPT and U7 hairpin variants that further boosted RNA editing relative to the original SmOPT and U7 hairpin. Coupling our improved SmOPT and U7 hairpin variants to gRNAs resulted in up to 76% targeted editing from a single integrated DNA copy of the gRNA construct, representing a 10-100-fold increase over previous approaches. Furthermore, our new SmOPT U7 variants also show promise for non-ADAR based therapies, increasing the efficiency of published antisense constructs for DMD exon skipping by several fold in differentiated myoblasts. In summary, our improved snRNA framework consistently enables high efficiency programmable RNA editing across many target sites under endogenous ADAR levels. These results demonstrate that our novel SmOPT and U7 hairpin variants provide great promise as a universal scaffold for ADAR-based RNA editing as well as other antisense RNA therapies.

334 Improving the Precision and Fidelity of Cas9 and PEn Mediated Gene Editing through Pharmacological Treatment

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Genome editing tools, particularly CRISPR/Cas9-based methods, have revolutionized biomedical research and created possibilities for developing curative treatments for genetic disorders. Despite rapid advancements, low efficiency of targeted DNA integration and generation of unintended mutations remain significant barriers for genome editing applications. These issues stem from the interplay between Homology-Directed Repair (HDR), Non-Homologous End Joining (NHEJ), and Microhomology-Mediated End Joining (MMEJ) DNA Double-Strand Break (DSB) repair pathways. To enhance the efficiency of CRISPR-Cas9 genome editing, we developed pharmacological treatments to improve the efficiency of Cas9 and Prime Editing nucleases (PEn) mediated knock-in. Specifically, treatment with AZD7648 and Pol-Theta inhibitors (referred to as 2iHDR) significantly enhanced HDR-mediated insertion precision, with efficiencies of up to 80% and nearly no generation of unwanted Insertion-Deletions (InDels). AZD7648 treatment also increased PEn-mediated insertion precision and reduced InDels. Importantly, these strategies reduced Cas9-related off-target effects, significantly enhancing the performance and accuracy of CRISPR-Cas9 gene editing and primed insertions.

335 Nuclease-Free Gene Editing with Peptide Nucleic Acids: A New Class of *In Vivo* Gene Editors

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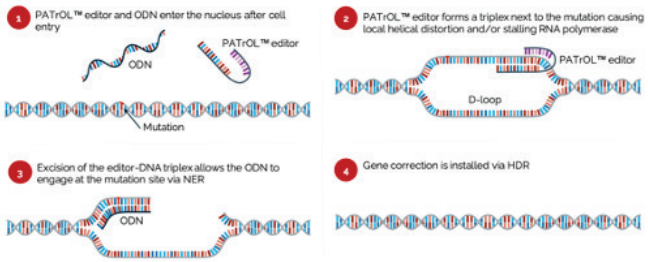
Gene editing promises permanent cures to genetic diseases. Early generations of gene editing technologies (e.g., generations of CRISPR/Cas and base editors) have suffered from fidelity (off-target and by-stander edits) and toxicity (including double stranded breaks) issues, which may limit their ability to be successfully administered *in vivo*. Additionally, these first-generation editors have a requirement for a protospacer adjacent motif (PAM) sequence directly adjacent to the causal variant of interest and are constrained to gene disruption or editing transition mutations, meaning the total addressable market of all such editors, combined, is ~20% of known genetic mutations in ClinVar. The introduction of prime editing (PE) resolves some of the limitations of these earlier technologies, by relaxing the PAM sequence constraint and allowing editing of transversions, insertions, and deletions such that an estimated 90% of all genetic mutations are now technically addressable. Challenges that remain include the ability to be flexible in delivery approaches such that editors can be delivered to a variety of tissues that manifest pathologies, improving fidelity to maximize safety, and overcoming the likely acquired immunogenicity of a bacterial nuclease which could limit the ability to re-dose a patient to address tissue turnover. We have developed a technology platform, PATrOL™, comprised of modified peptide nucleic acids (PNAs). These editors are PAM-sequence unrestricted, can address all mutational types, have fidelity rates on the order of background mutational rates in human cells, and which have additional advantages over PEs in that we have flexibility in delivery and do not elicit an acquired immune response. PATrOL™ editors engage the double-stranded genome with single base selectivity to recruit endogenous nucleotide excision repair (NER) and homology directed repair (HDR) enzymes which replace the disease-causing mutation with the correct sequence. This approach uses multiple layers of sequence selectivity for a locus of interest and relies on the body's own endogenous high-fidelity machinery to reduce off target editing to well below that reported for BEs. The editors themselves are on the order of 100 nucleobases in total length and are charge-tunable to allow use with various delivery technologies. Finally, due to the synthetic nature of the editors themselves, there has not been an acquired response reported to date. In combination with non-immunogenic lipid nanoparticle delivery, the PATrOL™ editors have the potential to unlock repeat dosing *in vivo* to address tissue turnover and achieve clinically relevant editing efficiencies. Data will be presented that describes the performance of our nuclease-free editing platform and how the technology in being applied to address several monogenic diseases that remain of high unmet need.



336 Compact Engineered Human Mechanosensitive Transactivation Modules Enable Potent and Versatile Synthetic Transcriptional Control

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Gene and cell therapies can be broadly enabled using engineered transactivation domains (TADs) combined with programmable DNA binding platforms. Despite recent progress in programmable CRISPR/Cas-based transactivation (CRISPRa) technologies, the TADs used in these systems often contain viral elements and/or are prohibitively large for many applications. Here we defined and optimized minimal TADs built from human mechanosensitive transcription factors (MTFs). We used these components to construct potent and compact multipartite transactivation modules (MSN, NMS, and eN3x9) and to build the CRISPR-dCas9 recruited enhanced activation module (CRISPR-DREAM) platform. We found that CRISPR-DREAM was specific, robust across mammalian cell types, and efficiently stimulated transcription from diverse regulatory loci. We also showed that MSN and NMS were portable across Type I, II, and V CRISPR systems, TALEs, and ZF proteins. Further, as a proof of concepts, we used dCas9-NMS to efficiently reprogram human fibroblasts into iPSCs and demonstrated that MTF TADs are efficacious and well tolerated in therapeutically important primary human cell types. Finally, we leveraged the compact and potent features of these engineered TADs to build new dual and all-in-one CRISPRa AAV systems. Altogether, these compact human TADs, fusion modules, and new delivery architectures should be valuable for synthetic transcriptional control in a wide range of gene and cell therapy and assocated biomedical applications.

337 CRISPR Assisted Trans-Splicing of RNA Fragments

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We introduce a CRISPR-based RNA editing approach that hijacks the cellular spliceosomal machinery to promote trans-splicing between an endogenous target pre-mRNA and a recombinant RNA fragment. To achieve such, a catalytically dead Cas13 nuclease (dCas13) is paired with a recombinant trans-splicing RNA molecule. The latter is comprised of a Cas13 guide RNA linked to a hemi-intronic sequence and one or more recombinant exons. The resulting ribonucleoprotein complex is guided to an endogenous target pre-mRNA yielding a chimeric messenger RNA via trans-splicing. This approach, referred to herein as CRISPR Assisted Fragment Trans-splicing (CRAFT), can be utilized to replace one or more exons in the 5' or 3' region of any endogenous mRNA. We demonstrate robust CRAFT mediated mRNA editing across multiple targets within the transcriptome. Further, we describe a rigorous fluorescence-based screen to optimize guide selection. CRAFT is a broadly applicable and versatile RNA editing platform, with potential applications as a research tool for interrogating RNA biology and for therapeutic applications.

338 Precise Cut-and-Paste DNA Insertion Using Engineered Type V-K CRISPR-Associated Transposases

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CRISPR-associated transposases (CASTs) enable recombination-independent, multi-kilobase DNA insertions at RNA-programmed genomic locations. However, the utility of type V-K CASTs is hindered by high off-target integration and a transposition mechanism that results in a mixture of desired simple cargo insertions and undesired plasmid cointegrate products. Here we overcome both limitations by engineering new CASTs with improved integration product purity and genome-wide specificity. To do so, we engineered a nicking homing endonuclease fusion to TnsB (named HELIX) to restore the 5´ nicking capability needed for cargo excision on the DNA donor. HELIX enables cut-and-paste DNA insertion with up to 99.4% simple insertion product purity, while retaining robust integration efficiencies on genomic targets. HELIX has substantially higher on-target specificity than canonical CASTs, and we identify several novel factors that further regulate targeted and genome-wide integration. Finally, we extend HELIX to other type V-K orthologs and demonstrate the feasibility of HELIX-mediated integration in human cell contexts.

339 *De Novo* Design of 26S Proteasome Recruiters with Language Model-Derived Guide Peptides

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The human proteome consists of around 20,000 proteins, many of which can acquire or present pathogenic consequences in disease states. A subset of these are considered "undruggable" by standard pharmacological techniques using small molecules, due to their unstable structural conformations, localizations, and/or post-translational modifications. Targeted protein degradation (TPD) represents a powerful modality to eliminate pathogenic proteins via the ubiquitin-proteasomal system. However, small molecule-based TPD platforms, such as PROTACs and molecular glues, rely on accessible binding sites on structured protein targets. In this study, we leverage robust protein language models, trained on millions of natural amino

acid sequences to grasp key biochemical, structural, functional features of input protein sequences, to generate target-specific "guide" peptides specific to pathogenic proteins of interest. As a genetically-encoded PROTAC analog, we fuse these guide peptides to 26S proteasome recruiters, and demonstrate robust degradation of diverse target proteins in human cells, including membrane proteins, transcription factors, and fusion oncoproteins. We further demonstrate that our constructs exhibit high specificity via whole-cell proteomics, and can effectively degrade oncogenic drivers, including β-catenin in colorectal cancer cells and EWS-FLI1 in Ewing sarcoma cellular models. Overall, our compact, programmable, genetically-encoded degradation strategy presents exciting opportunities for downstream in vivo studies and clinical translation.

Ophthalmic and Auditory Diseases

340 A Mutation-Independent RNA Replacement Approach Based on a RHO-Targeting Trans-Splicing Ribozyme for the Treatment of Autosomal Dominant Retinitis Pigmentosa (adRP)

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Retinitis Pigmentosa (RP) is the most common hereditary degenerative eye disease and caused by abnormalities in retinal photoreceptors (rods and cones) or the retinal pigment epithelium (RPE). Rhodopsin mutations account for about 25~30% of adRP. Although a single Pro23His (P23H) mutation in the rhodopsin (RHO) gene is currently known to be the most common cause of adRP in North Americans, over 150 autosomal dominant mutations also have been identified in the *RHO* gene. Group I intron-based trans-spicing ribozyme reprograms target RNA into a gene of interest through RNA replacement. In this study, we developed a specific trans-splicing ribozyme in a mutation-independent therapeutic strategy that can replace, and thus edit endogenous RHO RNA with exogenous functional RHO RNA and delivered it using adeno-associated virus (AAV). The most accessible target site of rhodopsin RNA was identified by RNA mapping, using a ribozyme library. Sequencing analysis of trans-splicing reaction sites revealed that the most efficient target site occurred at the base (U) in RHO RNA 5' UTR. To improve the trans-splicing specificity and efficiency of the ribozyme, we modified and optimized the structure of the ribozyme targeting the most accessible site of target RHO RNA. The candidate with the highest trans-splicing efficiency was then selected through comparative analysis in vitro using cells. Of note, we observed that various mutant RHO RNAs were trans-spliced with high fidelity and replaced with wild-type (WT) RHO RNA by the RHO-targeting ribozyme in cells. To verify in vivo function, an AAV vector, encoding the optimal RHO-targeting ribozyme, was constructed and bilaterally delivered by subretinal injection into the eye of P23H human RHO (hRHO) knock-in mice at postnatal week 5. Control groups are untreated or bilaterally PBS-treated knock-in mice. We evaluated rod isolated retinal function using the electroretinogram (ERG) in AAV-treated and control mice up to 26 week post injection (wpi). In contrast to controls, the b-wave amplitudes of AAV-treated eyes showed significant and dose-dependent increases which were maintained until 26 wpi. Molecular and cellular analysis of retina tissue showed that P23H RNA was replaced with WT RHO with high efficacy and fidelity and the outer nuclear layer (ONL) appeared significantly thicker in the RHO-targeting ribozyme treated areas in eyes. In addition, we delivered AAV encoding the RHO-targeting ribozyme via subretinal injection to both eyes of TgP23H hRHO pigs between postnatal days 3 to 7. From birth, untreated TgP23H hRHO pigs have no rod-isolated ffERG response. Eyes injected with AAV gained a significant rod-isolated ffERG response across all viral titers, first observed at 8wpi and lasting through 28wpi. Within the RHO-targeting ribozyme treated areas, Tg rods had elongated inner and outer segments (IS/OS) and thicker ONL compared with untreated areas in the same eye and the untreated/vehicle-treated eyes. Here, we proved that the ribozyme effectively prevents rod photoreceptor degeneration and preserves their function in both P23H hRHO knock-in mice and adRP TgP23H hRHO pig models through specifically targeting and trans-splicing of target RHO RNA. Taken together, these results suggest that RNA replacement based on RHO-targeting ribozymes could be a potential mutation-independent therapeutic strategy for RHO-adRP patients.

341 Massively Parallel and Systematic Engineering Platform for Highly Compact, Cell-Type Specific, and Potent Smart Sensor Promoters for Precision Retinal Gene Therapies

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Adeno-associated viral (AAV) gene therapy is increasingly becoming an important therapeutic modality for ocular diseases, with demonstrated efficiency of gene delivery and relative lack of toxicity. However, a risk of toxic host-eye responses has been observed with some AAV vectors. A contributing factor may be promoter promiscuity that leads to AAV-mediated transgene expression in off-target cells, such as the retinal pigment epithelium (RPE). Ectopic expression of a therapeutic transgene may be a trigger for cell stress and/or inflammatory responses [5]. With the aim of satisfying the critical needs of both safety and efficacy, we generated compact, synthetic Smart Sensor promoters that are highly active in photoreceptor cells and relatively silent in RPE cells. We pursued two design approaches in parallel: 1)

high-throughput pooled screening of short, fully synthetic promoters and 2) clonal screening of longer elements derived from endogenous promoters and putative enhancers. For the first approach, we designed a pooled promoter library using transcription factor binding sites (TFBSs) identified through bioinformatics analysis of RNA-Seq datasets of primary cells. Tandem arrays of combinatorially arranged TFBSs were synthesized and screened as a pool in a Massively Parallel Reporter Assay (MPRA) in surrogate cell lines selected for both the ON target cell type (Y79 for photoreceptors) and the OFF-target cell type (ARPE-19 for RPE cells). For the second approach, differentially regulated promoters and enhancers, identified through bioinformatics analysis of RNA-Seq and DNase-Seq datasets, respectively, were cloned and screened individually by transient transfection. We developed a powerful analysis pipeline that leveraged the heterogeneity of individually transfected cells within a bulk population. Promoters and enhancers identified using this screen were systematically mutated to map core regulatory elements. The natural and synthetic core regulatory elements identified using these two approaches were combined to form chimeric promoters, yielding regulatory synergies that further boosted the strength and specificity of gene expression. This multi-pronged strategy yielded photoreceptor-specific synthetic promoters that achieve 100 to 10,000-fold specificity for the photoreceptor surrogate line Y79 over ARPE-19 (RPE), with expression levels equivalent to the strong constitutive CAG promoter currently in clinical use. All these potent synthetic promoters were 500 bp or less in length. Additionally, this effort uncovered highly compact promoters that retained strong selectivity for photoreceptors, albeit with reduced potency. Some of these compact promoters, as small as 130 bp, may be applicable to expression cassettes encoding large transgenes. These results validate our massively parallel and systematic workflow for designing highly compact, specific, and potent synthetic Smart Sensor promoters, which can be applied across various cell types and diseases of interest.

5. Xiong, W.; Wu, D.M.; Xue, Y.; Wang, S.K.; Chung, M.J.; Ji, X.; Rana, P.; Zhao, S.R.; Mai, S.; Cepko, C.L. AAV cis-regulatory sequences are correlated with ocular toxicity. Proc. Natl. Acad. Sci. USA 2019, 116, 5785-5794.

342 Rescue of *OTOF* Q829X Mutation-Induced Hearing Loss by *In Vivo* Delivery of Mini-dCas13X-Derived RNA Base Editor

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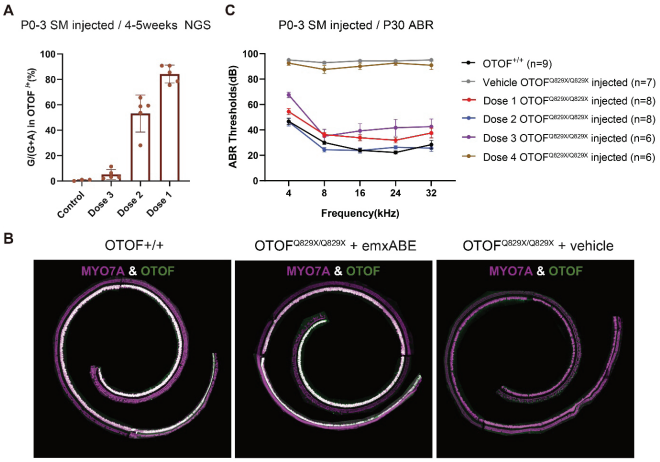
Background: Hearing loss is one of the most common sensory disorders that affects approximately 5% of the world's population, which causes great burden to the society. Recessive deafness is mostly congenital and profound, and accounts for ~80% of all hearing impairment. Mutations in the otoferlin (*OTOF*) gene cause prelingual and profound recessive hearing loss. Nonsense mutation c.2485C>T (p. Q829X) in the exon22 of *OTOF* gene is responsible for about 3% of all cases of recessive prelingual deafness in the Spanish. Although hearing aids can provide significant benefits to patients and cochlear implantation is more suitable for severe-to-profound hearing loss to restore sound transduction, there is still unmet medical need for a one-time, precise, and permanent strategy to potentially cure the genetic hearing loss. Here we report a small size and low off-target RNA base editing tool (emxABE) to achieve high A-to-I conversion and restore hearing loss by *in vivo* AAV delivery of mini-dCas13X-derived RNA base editor (AAV-gOTOF-emxABE) to OTOF$^{Q829X/Q829X}$ mouse model. **Methods**: We constructed a humanized OTOF-Q829X mouse model, with part of the mouse *Otof* gene sequence replaced with human *OTOF* gene sequence containing the Q829X mutation. To achieve a better therapeutic effect, we first optimized and improved the editing efficiency of the mini-dCas13X-derived RNA adenine base editor (mxABE) on the premise of low off-target, and thus obtained the enhanced mxABE (emxABE) with higher editing efficiency. One AAV vector, comprising gRNA targeting *OTOF* (gOTOF) and emxABE, was constructed to develop a therapeutic strategy that restores the hearing function by conversing TAG to TGG, restoring the expression of OTOF protein by read-through of a premature stop codon. Postnatal day 0-3 (P0-3) humanized OTOF$^{Q829X/Q829X}$ mice were injected through scala media (SM). We further tested AAV-gOTOF-emxABE base editing strategy for the treatment of P30 humanized OTOF$^{Q829X/Q829X}$ mouse model by injecting AAVs through round window. The editing efficiency was tested by deep sequencing. The OTOF protein was detected by immunostaining and hearing function was measured by auditory brainstem response (ABR). The toxicity of emxABE tool was evaluated by hematoxylin-eosin staining of the cochlear. **Results**: emxABE achieved >80% A-to-I conversion efficiency at the RNA level in the P0-3 humanized OTOF$^{Q829X/Q829X}$ mice injected with AAV-gOTOF-emxABE (Fig. 1A), with >90% otoferlin expression restoration in inner hair cells (Fig. 1B). Auditory function was restored to the wild type range (Fig. 1C) and was observed up to 8 months post AAV-gOTOF-emxABE injection in OTOF$^{Q829X/Q829X}$ mice. In addition, no toxicity was observed in the treated OTOF$^{Q829X/Q829X}$ mice. Restoration of auditory function was also observed in OTOF$^{Q829X/Q829X}$ mice injected with AAV-gOTOF-emxABE at P5-7 and P30. **Conclusions**: Our findings not only provided a preferential clinical strategy for the treatment of OTOF-Q829X induced hearing loss, but also suggested that base editors as the promising toolkits may be a potential strategy for the treatment of genetic hearing loss or other kind of diseases in the future. To the best of our knowledge, we are the only group using base-editor strategy to potentially cure the genetic hearing loss caused by OTOF mutation.



343 Indirect Comparison of Lenadogene Nolparvovec Gene Therapy versus Natural History in m.11778mt-ND4 Leber Hereditary Optic Neuropathy Patients

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Introduction: Lenadogene nolparvovec is a promising gene therapy for patients with Leber hereditary optic neuropathy (LHON) due to the m.11778G>A *MT-ND4* mutation. The purpose of this analysis was to compare the visual acuity of *MT-ND4* LHON patients treated with lenadogene nolparvovec in clinical trials to the spontaneous evolution of visual acuity in an external control group of untreated *MTND4* LHON patients. A previous analysis was performed on treated patients from three phase 3 studies. This updated analysis incorporates a fourth phase 3 trial, REFLECT, in which patients received lenadogene nolparvovec unilaterally or bilaterally. **Methods:** Individual visual acuity data of 174 *MT-ND4* LHON patients intravitreally injected in one or both eyes were pooled from four phase 3 trials: REVERSE, RESCUE, RESTORE and REFLECT. The external control group included 208 age-comparable (≥ 15 years old) untreated m.11778G>A *MT-ND4* LHON patients from 11 natural history studies. **Results:** Both cohorts were predominantly male patients (81.2%) with a median age at onset of vision loss of 26.0 years. Eyes treated with lenadogene nolparvovec had better visual acuity at all timepoints when compared to natural history eyes. Mean [95% confidence interval (CI)] difference versus natural history was 0.30 [0.39; 0.22] LogMAR (+15 EDTRS letters equivalent) at last observation ($p<0.01$) with a maximal follow-up of 3.9 years after treatment. When adjusting for covariates of interest (gender, age of onset, ethnicity, and duration of follow-up), the estimated mean [95% CI] difference was 0.43 [0.53; 0.33] LogMAR (+21.5 EDTRS letters) versus natural history at last observation ($p<0.0001$). Most treated eyes were on-chart (LogMAR ≤ 1.6) compared to less than half of natural history eyes at last observation (76.1% versus 44.4%; $p<0.01$). The treatment effect was larger in eyes of patients who received bilateral treatment than those of patients who received unilateral treatment. **Conclusion:** This comparison of treated patients with natural history patients confirmed a clinically meaningful and sustained improvement in visual acuity induced by lenadogene nolparvovec intravitreal injection in m.11778G>A *MT-ND4* LHON patients.

344 Development of a Mutation Independent Gene Therapy for Cone Reactivation in the Treatment of Retinitis Pigmentosa

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We are developing genomic medicines for inherited retinal disorders and in particular adeno-associated virus-based gene therapies, independent of causative mutations. Our lead product SPVN06, aiming to improve cone outer segment renewal and viability at intermediate stages of the disease, has recently been approved for human clinical studies. SPVN20, currently in preclinical evaluation, is targeting cone photoreceptor cells at later stage of the disease when remaining cells have lost phototransduction function. Compared to other vision restoration strategies, SPVN20's mechanism of action would be compatible with restoration of color vision. GIRK1 is a G-protein gated inward rectifier potassium channel. This G-protein gated channel is originally found in the brain and the heart and controls neuronal excitability through hyperpolarization. This is achieved through co-expression of GIRK1 with other GIRK family members by forming heterotetramers. A mutant of GIRK1, referred to as GIRK1 F137S, forms functional homotetramers. In a human cell line (HEK), we first showed that when co-expressed with an opsin, human GIRK1 F137S (a G protein sensitive channel) generates a current upon light stimuli. These light-induced responses were measured by patch clamp recordings in vitro. We then asked whether GIRK1 F137S could be expressed in degenerating cones and improve their function in mouse models of RP. We optimized our expression cassette containing GIRK1 F137S transgene and chose PR1.7 cone specific promoter to control its expression. We treated rd10/rd10 mice, a rapid retinal degeneration mouse model. Subretinal administration of AAV8-GIRK1 F137S led to significant -but transient- functional improvements at 3 weeks after administration. Using immunohistochemistry, we detected GIRK1 F137S protein at the cell membrane of cones in AAV-treated rd10 retinas. Visual improvements were transient likely due to the low number of cones remaining at the later timepoints in this fast-progressing model. At advanced RP stages, retina is thinner and subretinal injection risky. Therefore, a desirable injection route in patients is the intravitreal (IVT) delivery, which is noninvasive to the retina. To determine whether GIRK1 F137S could be delivered to human cones, we used an engineered IVT-permissive AAV2 variant (AAVi), optimized for transducing retinal cells when delivered intravitreally. We transduced cone-enriched human retinal organoids using AAVi-PR1.7-GIRK1 F137S-GFP. GIRK1 F137S expression was found in human cones by immunohistochemistry, at the cell membrane, as observed in rd10 mice. Altogether, these encouraging in vitro and in vivo findings in mouse and human tissue

support exploring the potential of AAVi-GIRK1 F137S, with the aim of developing a noninvasive and restorative gene therapy product for late-stage RP patients.

345 Precise Targeting of GJB2 Cells Resulted in Safe and Efficacious Gene Therapy in a Rodent Model of Hearing Loss Due to GJB2 Deficiency

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Background: Mutations in Gap junction beta protein 2 (GJB2) are the leading cause of non-syndromic, prelingual deafness (DFNB1) worldwide. A major challenge in developing a gene replacement therapy is the ability to replicate the endogenous expression pattern in the cochlea, as GJB2 is expressed in a variety of cell types at different expression levels. A successful DFNB1 gene therapy must provide benefit by sufficiently targeting the diverse set of GJB2-expressing nonsensory cells but also must avoid toxicity by excluding expression from critical sensory cells. Here we demonstrate that selective targeting of GJB2-expressing cells results in robust and durable hearing restoration in a translationally relevant mouse model of GJB2 deficiency. Methods: Adult or neonatal mice were injected via the posterior semicircular canal with AAV encoding GJB2 or GFP driven by a ubiquitous promoter or GJB2 regulatory elements. Hearing was assessed using functional measures of auditory function (auditory brainstem response, otoacoustic emissions). Animals were sacrificed and immunohistochemistry was performed to assess cochlear morphology and transgene expression. Results: We leveraged integrative bioinformatic analyses of bulk and single cell epigenomic datasets to design combinations of GJB2 proximal promoter/enhancer regions that could mirror the endogenous GJB2 expression pattern. Candidates were screened in neonatal cochlear explants, identifying a series of combinations which drive expression in various groups of GJB2-expressing cells while excluding expression from hair cells and neurons. The expression profiles of the top candidates were confirmed *in vivo* in both mouse and non-human primates. Next, we evaluated the ability of our promoter/enhancer designs to restore hearing in a GJB2 deficiency mouse model. We demonstrate that several of our regulatory elements were able to drive hearing restoration in human GJB2 injected ears compared to contralateral ears and naïve controls. We show that our top candidate led to stable and durable hearing recovery in >75% of injected animals, achieving wildtype thresholds in the majority of animals. Finally, we also show that GJB2 overexpression under the control of these regulatory elements is safe in wild-type animals, compared to using a ubiquitous promoter, which leads to hair cell death and elevated hearing thresholds. This confirms that we successfully eliminated off-target toxicity by appropriately restricting GJB2 transgene expression. Conclusions: Our results underscore the importance of using the proper regulatory elements to drive GJB2 expression in AAV-based gene therapy for the most common form of genetic deafness and highlight the power of bioinformatics to achieve a successful design. The robust and durable recovery observed in mice with our vector represents significant progress towards the development of a gene therapy to restore natural hearing to DFNB1 patients.

346 A Novel Stem Cell Therapy for Treating Patients with Retinitis Pigmentosa

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Stem cell therapies have shown great promise to restore vision in patients affected by retinal degenerative diseases. However, there are still challenges that remains due to the efficacy gap between *in vitro* studies and *in* vivo cell transplantation. This is partly due to the hostile microenvironments in which these cells are transplanted in. In a degenerative tissue, the host immune system and hostile conditions cause major challenges that need to be addressed.In retinitis pigmentosa (RP), the loss of vision is due to the death of both rod and cone photoreceptors. The absence of rods causes the loss of critical metabolic factors directly affecting cone survival and function. Therefore, cell therapies for RP are focusing on protecting, repairing, and replacing cone photoreceptors.To address this key issue, we have created a 3D hydrogel-based stem cell product that secretes neuroprotective factors in the vitreous to preserve cone photoreceptors. This biocompatible hydrogel mimics the *in vivo* extracellular matrix to offer a thriving environment for cellular proliferation and viability. Our biomaterial encapsulates a novel enriched target cell population that produces specific neurotrophic factors which are lost in RP: rod precursor cells. We have created a novel and proprietary protocol to isolate both rods and cones precursor cells in high purity and viability, allowing for high expansion capabilities.We have engineered this technology by combining material science and cell biology and have examined its effects *in vitro* on cone cells and *in vivo* with the rd1, and rd10 mice models along with a safety study in rabbits.Our studies show that our technology is a multimodal therapy that targets specific neurodegenerative pathways such as neuro-glycolysis and neuro-inflammation. The neurotropic factors present can enhance glucose uptake by cone photoreceptors and reduce the expression of W6/32 markers indicating a reduction in neuro-inflammation. Following the injection of our stem cell-matrix product in the vitreous of rd-1 mice (P26) we observed a 10-fold increase in cone survival (central retina) along with the presence of rods ay later time points (P60); these results translated into functional recovery in the rd-10 animal model. This technology is the first of its type to be gene agnostic and which can target multiple pathways that are affected in RP patients.

Gene Targeting and Gene Correction: CNS

347 High Efficiency *In Vivo* RNA Editing in the CNS with AAV-Delivered ADAR gRNAs

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RNA editing using guide RNAs (gRNAs) that recruit the endogenous adenosine deaminase acting on RNA (ADAR) enzyme has great potential as a versatile gene therapy approach, without the need to deliver a protein or protein-encoding transgene. ADAR deaminates adenosines into inosines, which are read as guanosines by the translational machinery, creating a functional A-to-G base change. The central nervous system (CNS) expresses high levels of ADAR1 and ADAR2, making it a particularly attractive target organ for therapeutic application of RNA editing. However, published results to date have relied on delivery of hyperactive forms of ADAR to achieve measurable in vivo RNA editing in the CNS. Here we demonstrate novel gRNA design combined with increased gRNA expression and stability that unlocks high efficiency RNA editing with endogenous ADAR in cell-based neuronal models and the CNS of adult mice. We began by applying our RNAfix gRNA discovery platform to CNS-relevant target adenosines. Briefly, RNAfix combines high throughput screening with generative machine learning to engineer gRNAs that interact with a target transcript, recruit endogenous ADAR and specifically edit the target adenosine. Therapeutic application of gRNAs requires sustained, high-level expression in target cells, leading us to choose a DNA-encoded delivery approach using AAV. We have previously shown that expressing gRNAs with elements of natural small nuclear RNAs (snRNAs) increases editing efficiency in multiple cell types. Importantly, in vivo delivery may only result in as low as one copy of the AAV genome per target cell, in contrast to in vitro systems that are amenable to high dose plasmid transfection or high AAV MOI transduction. Thus, maximizing gRNA expression and stability from each AAV genome is critical for high efficiency in vivo RNA editing. To this end, we undertook two engineering approaches: snRNA promoter engineering and AAV vector optimization. Initial experiments in HEK293 cells demonstrated that combining the enhanced promoters with improved vector designs resulted in up to 10-fold higher gRNA expression compared to starting designs. We then performed AAV transductions in differentiated SH-SY5Y cells, human NSC-derived neurons, mouse primary neurons and finally in adult mice. In vitro, the improved gRNA expression constructs increased target base editing efficiencies from <25% to >90%, with robust editing maintained at MOIs as low as one thousand viral genomes per cell. AAV vectors encoding the top construct designs were then injected into mice by intracerebroventricular delivery and bulk brain samples were collected four weeks post injection for RNA extraction and analysis. We observed up to 72% target adenosine editing in cortical tissue, far exceeding previously published data even with exogenous, hyperactive forms of ADAR. Given the potent and durable neuronal penetrance of AAV, our results support the delivery of RNAfix gRNAs in optimized expression constructs as a promising approach for treating a wide range of CNS diseases.

348 Targeting CX3CR1 Gene to Improve Microglia Reconstitution and Transgene Delivery into the CNS upon Hematopoietic Stem and Progenitor Cell Transplant

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Transplantation of hematopoietic stem/progenitor cells (HSPC) engineered by integrating vectors for expression of disease specific therapeutic transcripts has shown unprecedented curative potential in patients affected by monogenic neurometabolic diseases (NMD) when treated in early disease stages. This benefit is mediated by myeloid transplanted cell progeny in the central nervous system (CNS) that acts not only as vehicle for therapeutics, but also as modulator of neuroinflammation, possibly extending the application of this approach to a broader spectrum of neurodegenerative diseases (ND). Nonetheless, phenotypic effects after treatment are delayed likely due to the slow pace of CNS engraftment and differentiation of engineered HSPC into microglia like cells (MLC) compared to the rapid progression of neurodegeneration, that hampers the broad application of this approach. Here, we propose a novel gene addition strategy at CX3CR1 (CX) locus that may successfully address these limitations. CX gene encodes for a microglia chemokine receptor that regulates microglial recruitment to sites of neuroinflammation and microglia ontogeny. Firstly, we observed that transplantation of CX haploinsufficient (CXhaplo) HSPC resulted in a more robust engraftment and generation of MLC as compared to WT HSPC in competitive transplantations, where CXhaplo HSPC progeny prevailed over WT in the repopulation of hematopoietic organs (60% vs 40%) and brain (80% vs 20%). Next, to develop a therapeutic strategy combining the improved features of CXhaplo HSPC transplantation with a microglia specific transgene expression, we designed a CRISPR based gene addition strategy with a promoterless, splice trapping cassette encoding for a fluorescent reporter to be inserted into CX intron in a CX expressing cell line. We showed that targeted insertion allows regulated transgene expression and CX knockout only in HDR edited cells, while NHEJ indels did not impacted CX expression. We then used an established protocol based on Cas9/gRNA electroporation and AAV6 transduction to deliver the cassette in human HSPC (hHSPC), obtaining efficient integration (45% of the alleles) of the reporter gene, resulting in CX transcriptional and protein downregulation. Edited hHSPC were able to engraft into primary and secondary myeloablated immunodeficient mice, showing higher engraftment than AAVS1 edited hHSPC and increased transgene expression on human MLC compared to hematopoietic organs, as per physiologic CX expression. Interestingly, expression of the transgene driven by CX promoter in engrafted MLC was stronger than the expression driven by a conventional PGK promoter. Finally, a branching analysis performed on engrafted MLC revealed a greater extent of ramifications and

complexity of CXhaplo and CXedited *vs* WT and mock cells, both in the mouse- and human-into-mouse transplantation settings, suggesting a more rapid acquisition of a mature microglia like morphology and phenotype. To elucidate the biological mechanism underpinning this phenomenon, we performed a transcriptomic analysis at single cell resolution on FACS sorted MLC from competitively transplanted mice. Gene ontology analysis on signature genes revealed significant upregulation of Cdc42 signal transduction pathway in a cluster enriched with CXhaplo cells, that may account for greater activity in cytoskeleton rearrangement, cell motility and cell cycle progression explaining the quantitative and qualitative advantage observed. In conclusion, we identified and validated CX as a key target to enhance the ability of HSPC to generate MLC upon transplantation and allow specific and robust transgene expression in the CNS.

349 A Synthetic miRNA IFFL Circuit Module for Rett Syndrome Gene Therapy

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The precise control of gene expression can enhance our ability to ameliorate or cure disorders that exhibit gene dosage sensitivity. Rett Syndrome gene therapy is a prototypical example of such a 'Goldilocks problem', where the delivered gene MECP2 must be expressed, but too much expression is harmful, as in MECP2 duplication syndrome. Multiple factors, including cell-cell variation in viral vector uptake and high expression from synthetic promoters, can cause expression levels to extend beyond the therapeutic window. An ideal gene therapy would adapt to these factors to maintain expression at an engineered, therapeutic setpoint. An incoherent feedforward loop (IFFL) circuit could perform this adaptive function. Here we show that an IFFL can be implemented using a compact synthetic miRNA module that can be placed within an adeno-associated viral vector (AAV) without severely constraining the other cargo in size, and confirm that it can be used to regulate MECP2-EGFP gene expression in cell culture. We packaged the circuit into an engineered, CNS-targeting capsid AAV-CAP.B22, and delivered it systemically to mice. We compared ectopic and endogenous MECP2 expression using hybridization chain reaction (HCR) analysis to count mRNA transcripts in brain slices. While an unregulated expression cassette overexpressed MECP2 relative to the endogenous MECP2, the regulated cassette matched ectopic and endogenous gene expression levels. Altogether, these results demonstrate how synthetic miRNA IFFL circuits allow the precise control of gene therapies.

350 CRISPR-SKIP 2.0: A Highly Efficient Base Editing Toolbox for Exon-Skipping That Enables a Therapeutic Approach for Alzheimer's Disease

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CRISPR-SKIP is a technology that utilizes base editors to install precise mutations in splice acceptor sites of exons, preventing their recognition by the spliceosome and inducing skipping of the exon. Exon skipping has also been accomplished with antisense oligo nucleotides (ASOs), which continues to be explored in clinical trials, and some products are approved for use. However, CRISPR-SKIP provides several advantages over ASOs including the ability to introduce permanent modifications that provide a long-term benefit with a single administration. Nonetheless, multiple challenges prevent the widespread application of CRISPR-SKIP. First, the use of wild-type SpCas9 can only edit splice sites flanked by NGG PAM motifs, limiting the number of targetable exons as well as the amount of sgRNAs that can be screened to maximize efficiency. Second, the editing rates installed at many sites are relatively low. Third, cryptic splice sites in exons can cause the spliceosome to recognize alternative splice sites, leading to partial skipping, which may not be desirable. These limitations have prevented the therapeutic efficacy for high-value targets, such as *APP* exon 17, which contains the cleavage site for β-secretases, and its disruption could prevent the formation of toxic β-amyloid plaques in Alzheimer's disease. Specifically, while we have edited the splice acceptor of *APP* exon 17 with CRISPR-SKIP at low rates, we have not been able to accomplish exon skipping. To overcome these limitations, we developed a new toolbox for exon skipping, named CRISPR-SKIP 2.0, that utilizes cytosine and adenosine base editors with relaxed PAM requirements fused with newer generation deaminases, which, in combination, enable editing of essentially any target site with high efficiency. Compared to our previous technologies, CRISPR-SKIP 2.0 can induce DNA modifications at splice acceptor sites that were previously refractory to editing. Furthermore, to reduce the probability of unwanted cryptic splicing events, we implemented a new strategy consisting of simultaneous targeting of splice donor and acceptor sites, which not only reduced cryptic splicing but also enabled higher skipping efficiencies of targeted exons. In multiple instances, this technique enabled skipping of exons that were refractory to splicing modulation when editing the splice acceptor or donor alone. Importantly, CRISPR-SKIP 2.0 enabled the skipping of *APP* exon 17 in multiple human cell lines, and it also effectively reduced Aβ42 *in vitro* in a cell line used to model Alzheimer's disease. We also demonstrated that CRISPR-SKIP 2.0 possesses *in vivo* therapeutic potential as we delivered AAVrh10-packaged split adenine base editors via stereotaxic injections to the hippocampus of four-week-old mice carrying the humanized *APP* gene. This system efficiently edited the splice acceptor and donor sites within *APP* exon 17 one month after injection and induced 25% exon skipping within unsorted hippocampus tissue without introducing unwanted cryptic splicing. These results demonstrate that CRISPR-SKIP 2.0 can edit splice donors and acceptors and skipping of exons that were previously resistant

to skipping. Moreover, the simultaneous targeting of splice acceptor and donor sites demonstrated improved exon skipping as well as *in vivo* therapeutic potential in a mouse model of Alzheimer's disease, highlighting the potential impact of CRISPR-SKIP 2.0 in biomedicine and gene therapy.

351 Rewriting *ABCA4* RNA for the Treatment of Stargardt Disease

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Exon editing is a promising strategy for correcting disease-causing mutations by inducing trans-splicing between a therapeutic RNA Exon Editor molecule and an endogenous mutated pre-mRNA target, forming repaired mRNA and wild-type protein. Exon Editor RNA molecules can be designed to rewrite multiple contiguous exons carrying pathological mutations with the correct native sequence, thereby addressing the underlying genetic causes of disease. Potential applications include indications, such as Stargardt disease, which cannot be addressed by conventional gene therapies or gene editing approaches due to limited AAV packaging capacity, high mutational variance within gene targets, and/or narrow gene dosage indices. AAV-encoded RNA Exon Editors provide the durability of gene therapy, without the use of foreign enzymes, while maintaining endogenous gene expression patterns and thus, avoid many risks associated with direct DNA editing or gene replacement. Here we describe the process by which Ascidian's exon editing technology uses the endogenous spliceosome to rewrite mutated RNA resulting in full-length, correct mRNA molecules and protein rescue. We have applied this approach for the treatment of *ABCA4*-related retinopathies, including Stargardt Disease. We generated a high-throughput, multiplexed screening platform to design, evaluate, and optimize the RNA editing efficiency of thousands of Exon Editor molecules at a time. Here, we report on *ABCA4*-targeting Exon Editors that can efficiently replace 22 exons in the 5' half of the 7 kb *ABCA4* coding sequence, which covers 50-60% of known patient mutations, resulting in complete protein rescue *in vitro* in an engineered *ABCA4* mutant cell line. Next, we demonstrate durable and well-tolerated exon editing at therapeutically relevant levels in non-human primate retina following a single subretinal injection of AAV-encoded *ABCA4*-targeting Exon Editors. Moreover, we have recapitulated robust *ABCA4* exon editing in human donor-derived retinal explants following treatment with an AAV-encoded *ABCA4*-targeting Exon Editor. IND-enabling studies are underway to bring this program into the clinic, wherein we hope to demonstrate benefit to those living with *ABCA4*-related retinopathies. Finally, confirmation of the feasibility of Exon Editing has been further demonstrated in multiple other genetic targets *in vitro*. As we continue to work toward realizing the full potential of RNA exon editing and extending this approach to additional indications having high unmet medical need, this report demonstrates the translational viability of our therapeutic strategy.

352 Transcriptional Upregulation of α-L-iduronidase Utilizing Small-Activating RNA Following Bone Marrow Transplant in a Murine Model of Hurler Syndrome

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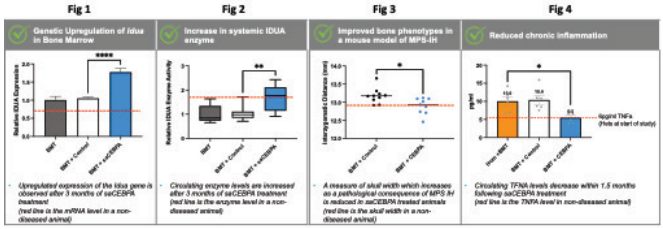
Hurler syndrome, or MPS I, is a genetic disorder caused by a deficiency in the lysosomal enzyme α-L-iduronidase (IDUA), resulting in cellular accumulation of the glycosaminoglycans heparan and dermatan sulfate. While gene therapy and editing technologies are on the horizon for disorders like MPS I, the standard of care for patients is currently enzyme replacement therapy (ERT) and hematopoietic stem cell transplant (HSCT). To date, a significant percentage of MPS I patients have received HSCT but still present with aspects of the disease, including skeletal dysplasia, cognitive decline, and chronic pain. For the patients who have previously received or are currently receiving these treatments, the options are limited for improving these outcomes. One potential approach to address this limitation is to increase IDUA enzyme levels produced by the transplanted cells and their progeny through transcriptional upregulation of the IDUA gene utilizing a small-activating RNA (saRNA). saRNAs are short, double-stranded RNA oligonucleotides designed to target the regulatory region of a gene and activate or increase expression at the transcriptional and epigenetic level. Currently, a saRNA-based treatment is in a clinical trial for hepatocellular carcinoma, where the saRNA is designed to upregulate the transcription factor CEBPA, the master regulator of myeloid cells. Because CEPBA is a transcription factor, it has many genomic targets, one of which is the promoter region of the IDUA gene. In the current study, it was hypothesized that following bone marrow transplant, MPS I mice treated with an saRNA targeting CEBPA (MTL-CEBPA) would increase *Idua* expression, and thus enzymatic activity. To this end, 5- to 6-week-old MPS I mice were irradiated and transplanted with bone marrow from sex-matched, wildtype mice harboring two functional copies of the *Idua* gene. Following engraftment, mice began receiving weekly intravenous injections of MTL-CEBPA, or a sham saRNA targeting firefly luciferase (Fluc), for up to 14 weeks. Compared to Fluc-treated mice, *Cebpa* mRNA levels in the bone marrow were moderately increased in mice receiving 0.5 mg/kg MTL-CEBPA ($p<0.05$) and trending toward significance in mice receiving 2.0 mg/kg MTL-CEBPA ($p=0.07$). In both cohorts receiving MTL-CEBPA, *Idua* mRNA levels were nearly two-fold higher than Fluc-treated mice (Fig. 1). This increased mRNA level also translated to a significant increase in systemic IDUA enzyme activity compared to Fluc-treated mice (Fig. 2). Phenotypic analysis demonstrated normalization of the inter-zygomatic distance in mice receiving MTL-CEBPA (Fig. 3). Further, CEBPA activation resulted in a significant reduction of circulating

TNFα was in MTL-CEBPA-treated mice (Fig. 4). These results demonstrate the power and flexibility that saRNA-based therapies can provide for patients who have previously received HSCT by transiently over-expressing the therapeutic gene in the engrafted and derived cells. This targeted approach by saRNA opens a new avenue of therapeutic development for not only lysosomal disorders such as MPS I, but all disorders where transcriptional activation or enhancement of a gene could provide therapeutic benefits.



353 Epigenome Therapy Approach to Tune *SNCA* Expression in Parkinson's Disease: Pre-Clinical Proof of Concept in PD Mouse Model

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Elevated *SNCA* levels are causative in Parkinson's Disease (PD) pathogenesis, while normal physiological levels of *SNCA* are essential to maintain neuronal function. Patients with *SNCA* triplication and duplication suffer from familial early onset from of PD, suggesting a therapeutics window of <30%. We aim to translate mechanistic knowledge of *SNCA* dysregulation towards the development of epigenome therapy for PD targeting *SNCA* expression. Towards this goal we developed *all-in-one* lentiviral vector (LV) carrying the deactivated CRISPR/(d)Cas9, a selected gRNA targeted at *SNCA*-intron1 and synthetic repressor molecules. Previously we provided *in vitro* proof-of-concept for the efficacy and efficiency of our LV-dCas9-repressor system in human iPSC-derived 'aged' dopaminergic neurons from a PD-patient with the *SNCA* triplication. We showed downregulation of *SNCA*-mRNA and protein levels that led to the rescue of disease-related pathological phenotypes including, mitochondrial dysfunction, neuronal-cell death, DNA damage and nuclear deficits. We now moved forward into *in vivo* validation studies. Our PD mouse model was generated by inducing the disease with an AAV-A53T-human SNCA vector that comprised of the mutated human-SNCA coding sequence fused with its native promoter/intron 1 region. We performed bilateral stereotactic injection of the AAV-A53T-human SNCA vector into the mouse substantia nigra (SN), the left SN was co-injected with the therapeutic LV-dCas9-repressor and the right SN was co-injected with the control inactive LV-dCas9 vector. Analysis of 10 mice demonstrated a significant reduction of 50% in human SNCA protein. Pathological examinations showed a significant robust reduction in Ser129-phosphorylated SNCA (nearly 80%) and in aggregated SNCA protein (70%). In addition, we observed significantly higher expression of tyrosine hydroxylase (TH +24%), suggesting a greater retention of dopaminergic neurons. We also collected safety measures. Monitoring daily behaviors showed no abnormalities in well-being criterions and no weight loss. In addition, safety measures demonstrated no issues for blood counts, serum chemistry and liver histology. In conclusion, our novel CRISPR/dCas9-based technology offers the unprecedented tool to modify a particular epigenetic mark resulting in effective fine-tuned reduction of *SNCA* expression levels sufficient for reversing PD-associated perturbations. This study provided an *in vivo proof-of-concept* for advancing our innovative epigenome editing-based system to a clinical trial as the next-generation PD epigenome therapy.

Gene and Cell Therapy Trials in Progress

354 Phase 1/2a Trial of Delandistrogene Moxeparvovec in Patients with DMD: 4-year Update

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Delandistrogene moxeparvovec (previously known as SRP-9001) is an investigational recombinant adeno-associated viral (rAAV) vector-based gene transfer therapy designed to compensate for missing dystrophin in Duchenne muscular dystrophy (DMD) by delivering the SRP-9001 dystrophin transgene, which encodes a shortened, engineered dystrophin protein that retains key functional domains of the wild-type protein. The objective of this Phase 1/2a, single-dose, open-label clinical trial (NCT03375164) is to evaluate the safety of systemic delivery of delandistrogene moxeparvovec in patients with DMD. Four ambulatory patients with DMD (≥4 to <8 years old) were enrolled. Patients were given an intravenous infusion of delandistrogene moxeparvovec at a dose of $2.0x10^{14}$ vg/kg (supercoiled quantitative polymerase chain reaction, linear plasmid standard equivalent of $1.33x10^{14}$ vg/kg) and prednisone (1 mg/kg/day) 1 day pre- to 30 days post-gene delivery. The primary outcome measure is safety. The secondary outcome measures include micro-dystrophin expression in pre- and post-muscle biopsies (Week 12 post-infusion). Key efficacy outcome measures include North Star Ambulatory Assessment (NSAA) and timed function tests. Previously, data from 3 years post-treatment were presented. Treatment-related adverse events were mild to moderate, occurred mostly in the first 90 days of treatment, and all resolved. No serious adverse events, study discontinuations, or adverse events associated with clinically relevant complement activation were reported. All patients demonstrated a clinically meaningful improvement on NSAA. Patients treated with delandistrogene moxeparvovec generally maintained muscle strength (Time to Rise and 4-stair Climb) and showed improvement in ambulation

ability (10-meter and 100-meter walk/run) from baseline to Year 3. The observed safety profile and the enduring response following treatment provide proof-of-concept for continuation of clinical trials assessing delandistrogene moxeparvovec using single-dose gene therapy in patients with DMD. We present the latest long-term (4-year) safety and functional data from this study. This study is funded by Sarepta Therapeutics.

355 Autologous Stem Cell Transplantation in HIV Infected Recipients Using Zinc-Finger Nuclease-Based CCR5 Edited CD34+ Cells: Persistence of Hematopoietic Progeny and Stem Cells at 5 Year Follow-Up

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We conducted the first clinical study in which gene-edited stem cells were administered to humans. A first-generation zinc-finger nuclease (ZFN) was used to edit CCR5 in mobilized autologous CD34+ stem cells (HSPC) and transplanted to persons living with HIV (PWH). A manufacturing process was qualified for HSPC selection and delivery of the ZFN mRNA by electroporation. In a feasibility and safety trial, eight healthy PWHs were infused with CCR5-edited (CCR5ed) HSPC, following non-myeloablative busulfan conditioning in two cohorts, cohort 1 targeted cumulative AUC 8,000 μM*min and cohort 2 12,000 μM*min. All manufactured CCR5ed-HSPC Drug Products met release specifications, with cell dosing of 2.2 - 11.5x10^6 CD34+ cells/Kg and CCR5 disruption of 8.0 - 43.3% (by miSeq assay). Sampling at the time of infusion showed high cell viability and purity, with presence of more primitive HSPC subsets. All patients engrafted, at 15 days for cohort 1 and 14 days for cohort 2. At 1 year (1Yr) post-transplant, CCR5 disruption was observed in peripheral blood (PB) mononuclear cells (MNC) in all patients (ranging from 0.07 - 2.95%), with levels stabilizing around month 6. Also, bone marrow (BM) analyses showed presence of CCR5 disruption in all patients, in purified CD34+ cells and in different cell subsets. Statistical analyses showed significant correlation between CCR5 disruption in BM CD34+ cells and busulfan exposure ($r=0.800$; $p=0.017$), which is particularly relevant considering the non-myeloablative conditioning. No significant correlation was seen between any features of the infused Drug Products and CCR5ed levels in BM CD34+ cells. Importantly, BM CD34+ CCR5 editing strongly correlated with CCR5ed levels in PB MNC ($r=0.968$; $p<0.0001$) and in total BM ($r=0.980$; $p<0.001$), and BM monocytes ($r=0.987$; $p<0.0001$) and other BM myeloid/immune cell lineages ($r=0.983$; $p<0.0001$), indicating successful engraftment of HSC carrying the CCR5 disruption. Also, significant correlation with CCR5ed levels in PB CD4+ T cells ($r=0.858$; $p=0.006$) was observed. Long-term follow-up demonstrated persistence of CCR5 editing in PB in all individuals at the most recent timepoint (years 4-5 post-infusion), maintaining significant correlation to the CCR5ed levels in BM CD34+ cells at 1Yr. In addition, in two individuals, BM studies were performed at ≥3 years post-infusion, and in both cases, again there was strong correlation between of CCR5ed levels in BM CD34+ cells and their frequency in PB MNC and CD4+ T cells and other BM cell subsets. Analysis of CD4 T cell recovery, immune function, memory subsets and TCR repertoire, as well as analysis of the HIV reservoir will be presented. In summary, we conducted the first long-term study in which HSPC were gene edited and infused in humans, without major adverse events. Engraftment of gene-edited stem cells was observed despite using a non-myeloablative conditioning and a first-generation editing construct. The persistence of CCR5ed progeny in all patients at 4-5 years post-infusion, with strong correlation to the levels of CCR5ed in the BM CD34+ cells post-transplant, supports the therapeutic use of ZFN gene-edited HSPC. With improved nuclease-based gene editing tools and transplant conditioning regimens, this strategy for gene editing in HIV warrants further research.

356 An Updated Follow-Up of BRL-101, CRISPR-Cas9-Mediated Gene Editing of the BCL11A Enhancer for Transfusion-Dependent β-thalassemia

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INTRODUCTION: β-Thalassemia is an inherited hemolytic disease that is prevalent worldwide. Over 200 mutations in the HBB gene, which encodes adult hemoglobin (HbA), result in β-thalassemia. Hereditary persistence of fetal hemoglobin (HbF) can alleviate the symptoms of anemia. CRISPR-Cas9-mediated disruption of the BCL11A erythroid enhancer results in the reduction of BCL11A expression and the induction of fetal γ-globin, which is a practicable therapeutic strategy for treating transfusion-dependent β-thalassemia (TDT). **METHODS**: We conducted a nonrandomized, single-dose, open-label clinical trial of BRL-101 in pediatric patients with TDT at Xiangya Hospital (NCT04211480) and 923rd Hospital (NCTO4205435). The inclusion criteria included age 5-15 years, clinically diagnosed as β-thalassemia major, phenotypes including β0/β0, β+/β0, βE/β0 genotype, and subject's body condition eligible for autologous stem cell transplantation. CD34+ HSPCs were edited with CRISPR-Cas9 RNP at the +58 erythroid specific enhancer region of the BCL11A gene. Patients were monitored for routine blood test, editing frequency, total Hb, Hb fractions on HPLC, F-cell percentage (circulating erythrocytes with detectable levels of HbF) and AEs. **RESULTS**: All 6 patients, including 4 β0/β0 phenotype patients, received BRL-101 treatment and successfully achieved transfusion independence. The neutrophil engraftment occurred within 1 month

for all patients, the platelet engraftment and last pRBC transfusions occurred within 2 months for two-thirds of patients (table 1). The total Hb levels began to increase steadily until they reached healthy levels, and HbF levels were elevated sustainedly (figure 1). F-cell expression increased to more than 90% within 6 months (figure 2). The CRISPR-Cas9-edited HSPCs engrafted and differentiated into multiple lineages that retained the gene editing. Editing frequency of PBMCs from patients increased to more than 60% during the follow-up periods (figure 3). No drug-related AEs leaded to study withdrawal or death of patients during treatment. The majority of AEs were consistent with that of mobilization, apheresis, myeloablation, and autologous hematopoietic stem cell transplantation. All of these AEs have been resolved. Four SAEs were reported during treatment, including left cheek soft tissue infection, acute upper respiratory infection, infective fever and thrombocytopenia. Only thrombocytopenia was attributed to BRL-101 and was gradually relieved. **CONCLUSIONS**: All 6 patients with different genotypes achieved transfusion independence, including the patients with the most severe β0/β0 phenotype. After BRL-101 infusion, the levels of total Hb and HbF increased significantly, and gene editing is retained in multiple lineages of HSPCs. The safety profile of BRL-101 is generally consistent with mobilization, apheresis, myeloablation and autologous hematopoietic stem cell transplantation. This study demonstrate that BRL-101 is a safe, potential functional cure for the treatment of TDT.

Table 1. Characteristics of patients and gene-editing outcomes

Patient No.	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	5	4	5	7	6	5
Sex	Male	Male	Male	Female	Male	Male
Genotype	41-42M1TM [IVS1-]	[845M]0-2850 [β+/β+]	β-28/41-42 [β+/β0]	β41-42/41-42 [β0/β0]	41-42/IVS-1-1 [β0/β0]	41-42/71-72 [β0/β0]
Race	Asian-Chinese	Asian-Chinese	Asian-Chinese	Asian-Chinese	Asian-Chinese	Asian-Chinese
Transfusion frequency (days) and volume before transplantation (U)	-20, 2	-15-20, 2.3	-15-20, 2.3	-15-30, 2	-15-20, 1.5	-25, 3
Number of CD34+ cells in infusion (per kg)	11.8×10^6	3.89×10^6	5.2×10^6	4.4×10^6	6.3×10^6	5.5×10^6
Neutrophil implantation time (days after transplantation)	14	17	29	24	19	27
Platelet implantation time (days after transplantation)	52	40	165	148	24	41
Time of last blood transfusion after transplantation	Day 29	Day 19	Day 114	Day 96	Day 26	Day 46

Figure 1. Substantial increases in total Hb and HbF level.

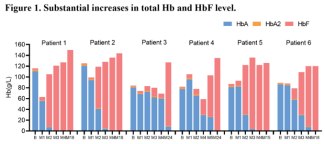

Figure 2. Durable increases in percentage of HbF.

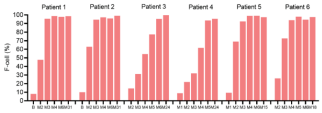

Figure 3. PBMCs from patients retained the gene editing.



357 Supraphysiological Enzyme and Early Neurocognitive Outcomes after Autologous Hematopoietic Stem Cell Gene Therapy in Patients with Mucopolysaccharidosis IIIA

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Ex vivo hematopoietic stem cell gene therapy (HSC-GT) provides a potential therapy for diseases with a single enzyme deficiency, where allogeneic HSC is ineffective. Mucopolysaccharidosis type IIIA (MPSIIIA) is a lysosomal storage disorder where pathogenic mutations in the *SGSH* gene result in the accumulation of heparan sulfate in all cells of the body. Build-up particularly affects the central nervous system, with devastating consequences. As the disease progresses, loss of previously acquired skills such as speaking and walking occurs, with profound behavioural disturbances and death between 10-20 years of age. There are no approved treatments. HSC-GT is expected to provide sufficient body-wide enzyme and potentially allow the trafficking of modified monocytes into the brain. Our phase I/II clinical trial (NCT04201405/EudraCT#2019-002051-42) has treated five children with severe MPSIIIA, aged 6-24 months. Recruitment to the trial is complete. Additional a patient was treated off-trial at 30 months old on a compassionate basis. Patient-specific HSCs were mobilised for transduction *ex-vivo* with a lentiviral vector expressing the *SGSH* gene under the myeloid CD11b promoter and cryopreserved. Patients received full myeloablative conditioning before product infusion. All trial patients had a vector copy number of 1.19-8.91 copies/cell/product, with a cell dose of 4.3-22.7x10^6 CD34$^+$/kg. Engraftment is sustained at >24 months (n=3), 18 months (n=1), or 9 months (n=1) follow-up, with supraphysiological expression of SGSH enzyme rapidly measurable in leukocytes (48 to 151-fold above median normal range by one month post-GT). Supraphysiological SGSH was also detected in CD3$^+$, CD19$^+$ and CD15$^+$ lineages and plasma. Cerebrospinal fluid (CSF) SGSH enzyme, previously undetectable, was within or above normal range by six months post-GT. Abnormal heparan sulfate storage at baseline was rapidly reduced in CSF, plasma and urine, with a normalization of urine glycosaminoglycan ratios. There has been no detection of replication competent lentivirus, and integration site analysis suggests a highly polyclonal product (9,771 to 70,686 unique integrations in leukocytes). No mappable unique integrations had a >1% contribution three-months post-GT or later, in samples analysed to date. Anti-SGSH IgG antibodies have been detected by one-month post-GT. However, there are no associated AEs and SGSH levels appear unaffected. Data from the off-trial patient (39 months post-GT) continues to show supraphysiological active SGSH, with neurocognitive outcomes suggesting an improved outcome compared to natural history. Neurocognitive assessments of trial patients are ongoing; early results show four out of five trial patients have gained skills in line with normal age equivalent development, one of whom shows an alteration in neurologic phenotype from natural history. In conclusion, treatment with *ex-vivo* autologous HSC-GT is well tolerated and delivers supraphysiological levels of enzyme throughout the body.

358 Initial Biomarker and Clinical Findings from the CAN*aspire* Canavan Disease Gene Therapy Trial: Exploration of Connections between NAA and Disease Severity

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Background: Canavan disease (CD) is an ultra-rare white matter disorder caused by mutations in the *ASPA* gene leading to accumulation of N-acetylaspartate (NAA) and profound early-onset impairment of psychomotor development. Although NAA levels are used diagnostically in CD, longitudinal data are lacking and the relationship between NAA, disease severity and progression are not understood. **Methods:** CAN*aspire* (NCT04998396) is a first-in-human open-label study evaluating the safety, pharmacodynamic (PD), and clinical activity of BBP-812, a systemically administered recombinant AAV9 h*ASPA* vector for the treatment of CD. Data from the CAN*inform* CD natural history study (NCT04126005) will be used as a comparator. As a PD marker of ASPA activity, NAA levels are quantified in urine and cerebrospinal fluid (CSF) by gas chromatography-mass spectrometry (GC-MS) and in brain by magnetic resonance spectroscopy (MRS). Clinical outcome measures include the disease-specific Canavan Disease Rating Scale (CDRS) that ranks the severity of 11 characteristic neurological and developmental features of CD, as well as performance-based and parent-reported pediatric motor and developmental scales. Effects on white matter pathology and brain volumes are followed by magnetic resonance imaging (MRI). Preliminary exploration of a potential relationship between NAA levels and disease severity was conducted using CAN*inform* natural history data, baseline findings in CAN*aspire* participants, and literature reports on NAA levels and phenotypic severity. **Results:** All 4 CANaspire participants dosed to date (median age at dosing 20.7 months, range 11.0-29.2 months) have shown decreases in urine, CSF and brain NAA. Urine NAA reductions (42-87%) exceeded the natural variability seen in CAN*inform* over the same duration of follow-up. Robust NAA decreases in CSF (70-90%) and brain MRS (up to 75%) were also observed across all participants with a strong correlation between MRS and urine levels (Spearman r =0.927). Data to date demonstrate persistent reduction in NAA levels in all compartments compared to baseline followed for 3 to 12 months post-treatment. CAN*aspire* participants have shown stabilization or improvement in total CDRS scores compared to baseline up to Month 12. Our preliminary data in CAN*aspire* participants indicate an association between NAA levels and CDRS total scores at baseline. This observation is consistent with prior literature and aligned with CAN*inform* natural history data supporting an association between NAA levels and disease severity. BBP-812 has been generally well tolerated; most adverse events (AEs) have been mild to moderate and mostly managed with adjustments in steroid dosing, with no treatment-related serious AEs. **Conclusions:** Preliminary data have supported the overall tolerability of BBP-812 and demonstrated robust and durable post-dose NAA reductions in urine, CSF and brain along with initial suggestions of clinical stabilization. While the literature and the ongoing natural history study suggest an association between NAA levels and phenotype, whether these early signs of PD and clinical activity translate to clinical efficacy awaits confirmation with additional participant data and longer follow-up in CAN*aspire*.

359 First in Human RESKUE Phase 1/2 Clinical Trial of Intravenous FBX-101 (AAVrh10.hGALC) Administered after Immune and Myeloablation for Unrelated Umbilical Cord Blood Transplantation Prevented Immune Responses, Increased GALC Activity, Restored Normal Brain Development, and Normalized Motor Function in Patients with Infantile Krabbe Disease

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Pre-immunity to AAV may be present in 50-60% of the population, which can lead to humoral and cellular responses against a capsid or therapeutic transgene. This has the potential to impact therapeutic efficacy and has recently been a focus for novel immune-modulation strategies to increase safety and possibly enable re-dosing approaches. Here we report clinical data utilizing a novel treatment regimen for patients with infantile Krabbe disease (IKD), a rapidly progressive demyelinating leukodystrophy due to deficiency of galactocerebrosidase (GALC). GALC deficiency leads to accumulation of psychosine which is toxic to oligodendrocytes and Schwann cells, resulting in demyelination and death by 2 years of age. IKD is currently treated with umbilical cord blood transplantation (UCBT), in which the immune system and the bone marrow are ablated to provide space for the UCBT, an immune stem cell source that is AAV naïve and can restore levels of GALC in the CNS. The approach avoids the antibody response to the capsid and transgene, allowing treatment of all patients including those patients who have no protein expression due to a null genotype. UCBT improves central nervous system (CNS) outcomes in asymptomatic infants, but motor function declines resulting in progressive peripheral neuropathy. The progression of peripheral neuropathy is presumed to be due to insufficient GALC as the patient grows. RESKUE is a first-in-human, open-label Phase 1/2 dose-escalating trial to evaluate safety and efficacy of FBX-101 after UCBT for the treatment of subjects with IKD. FBX-101 is an intravenously administered AAVrh10-hGALC vector designed to rescue peripheral nerve disease in patients with Krabbe disease that have previously received UCBT infusion. Nonclinical studies demonstrated that FBX-101 significantly increased survival and corrected peripheral neuropathy in Krabbe mice and dogs when given after HSCT. FBX-101 safety and biodistribution were established in a GLP rat toxicology study. Subjects (n=2) in cohort 1 received a single IV infusion of FBX-101

at a low dose ($3.0x10^{13}$ vg/kg) 25 and 29 days after UCBT infusion, respectively. FBX-101 was well tolerated, with no treatment-related serious adverse events (including no treatment-related liver enzyme elevations) observed up to Day 365 and Day 180, respectively. The subjects have engrafted with full chimerism. No antibodies to AAVrh10 have developed. We report a significant and sustained increase in plasma and CSF GALC enzyme activity following gene transfer and a reduction of psychosine levels. Subjects' motor skills, measured by PDMS-II, have improved and are within normal range. Finally, subjects have followed a normal white matter growth trajectory in the brain as measured by MRI-DTI. Administration of FBX-101 after UCBT represents a novel gene therapy strategy that leverages the immune- and myeloablation after UCBT, resulting in an immune environment that allows efficient AAV transduction. This administration strategy prevents antibodies against the transgene and capsid and ensures that sufficient GALC enzyme is delivered to sustainably support both brain and peripheral nerve development.

Wednesday Poster Session

361 Efficient Upper Motor Neuron Gene Delivery through AAV2-retro Spinal Cord Injection in Nonhuman Primate

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Introduction: Delivery of genetic payloads by viral vector administration into the spinal cord has significant potential to be of benefit for the treatment of diseases involving upper or lower motor neurons. For example, segmental neuroprotection delivered via virus injections into the C3-C5 of the spinal cord could result in preservation of diaphragmatic function and ventilation and protect upper motor neurons (UMN) in patients with Amyotrophic Lateral Sclerosis (ALS). Here we demonstrate efficient transduction of lower motor neurons (LMN) and UMN via direct delivery of AAVrg (also known as AAV2-retro) into the cervical spinal cord. Methods: Two adult male rhesus macaques (~5-8kg) that tested negative for pre-existing antibodies against AAV2 underwent multi-level cervical laminectomies at the C6-T1 segmental levels. Each animal received multiple (8-10) bilateral intraparenchymal injections of AAVrg vectors with engineered peptide insertion for opposite changes in binding of heparin and AAV receptor towards retrograde incorporation in motor neural circuit carrying different fluorescent reporter genes (mCherry or GFP) and the hSYN promoter. Head stabilization and a custom stereotaxis system were used for the procedures. Injections (2 ul deposit , delivered at 2 ul/min) were performed at a depth of 3mm, targeting the ventral horn of the spinal cord with a floating cannula. Euthanasia was performed six to eight weeks after the injections. Immunohistochemistry analysis was performed using antibodies against mCherry or GFP to demonstrate transduction of these proteins at terminal fields in the targeted area in the spinal cord and in neurons projecting to the injected areas.Results: All animals tolerated the procedures well and had no neurological deficits in the postoperative period. Injections resulted in robust transduction of neurons within the injected segments of the spinal cord with spread to the contralateral side. In addition, extensive neuronal transduction of correspondent corticospinal pyramidal neurons could be observed in the primary motor cortex, supplementary motor area and the pre-motor cortex, indicating retrograde axonal transport to the upper motor neurons projecting to the spinal cord.Conclusion: We found that pairing optimal promoter and viral vector serotype can result in efficient gene expression in the spinal cord and brain following direct delivery to the ventral horn of the cervical spinal cord. Delivery of protective genes to the upper motor neurons presents a challenge given their laminar distribution throughout the motor homunculus. The present study demonstrates that retrograde delivery of AAVrg to the primate upper motor neurons is a feasible and safe approach with critical advantages over diffuse delivery methods, such as the need for considerably lower doses of vector, convenient surgical access as well as the precise gene expression in a specific anatomical location. Characterization of retrograde transport and its limitations is important for increased understanding of the possibility of UMN restoration, which also provides contribution to trunk muscles function as a part of overcoming life-threatening respiration failure. Gene delivery into the spinal cord parenchyma is still an undervalued approach for segmental gene therapy, particularly in the cervical spinal cord, is a therapeutic outcome that could justify clinical translation in diseases such as ALS.Acknowledgement / Funding:This work was supported by the grants P50NS123103-01 and P51OD011132.

362 Limitations of Marmosets as an Animal Model for AAV Mediated Liver Gene Transfer

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Nonhuman primates are commonly used as an animal model to evaluate biodistribution and safety of Adeno-Associated Viral vectors (AAV). Cynomolgus macaques (*Macaca fascicularis*), an old-world monkey, and marmosets (*Callithrix jacchus)*, a new-world monkey species, have been used extensively in these preclinical studies. There are notable differences between these two species, especially in body weight, social behavior, and absence of CMAH (Cytidine monophosphate-N-acetylneuraminic acid hydroxylase) which has an important effect on the tropism of AAVs using sialic acid as the primary receptor. Marmosets are significantly lighter animals, making this species a desirable model especially for systemic administration of AAV due to reduced manufacturing cost. Here we compare the biodistribution profile of an AAV9.CAG.GFP vector intravenously (IV) administered in cynomolgus and marmoset monkeys. Prior to vector administration and during the course of the study, animals were immunosuppressed to limit immune responses to AAV capsid and GFP transgene. Animals

were sacrificed 28 days post vector administration and liver, cardiac and skeletal muscle tissues were collected and analyzed by ddPCR, RTddPCR and immunohistochemistry (IHC). Samples collected for IHC analysis were evaluated by a pathologist and percent of cells transduced reported. In addition, the prevalence of neutralizing antibodies (NAbs) to AAV in serum was evaluated in both species. The biodistribution data showed comparable transduction efficiency in cardiac muscle between species. Interestingly, liver transduction was significantly reduced in marmosets. Lower distribution in the liver of marmosets was also associated with lower gene expression of GFP measured by both RTddPCR and IHC. In addition, skeletal muscle transduction and gene expression in marmosets was greater than in cynomolgus macaques. Seroprevalence analysis showed that AAV NAbs in marmosets was significantly lower than in cynomolgus macaques. Most marmosets screened for AAV NAbs were seronegative. In contrast and as expected, high level of NAbs were detected in cynomolgus macaques. Although our study included a small number of animals (n=7) and a single vector dose (1e14 vg/Kg), our data indicate that marmosets may not be an ideal translational animal model to evaluate liver gene transfer and safety with administration of AAV vectors. AAV seroprevalence and liver transduction are significantly higher in cynomolgus macaques. The low levels of liver transduction observed in marmosets may not be predictive of toxicity observed in humans, especially with a high dose systemic AAV administration. Therefore, macaques may be a more representative translational model to evaluate liver transduction and safety for systemically administered AAV.

363 Dose-Dependent Cardiac Responses to Intravenous AAVrh.10hFXN Treatment of the MCK Murine Model of Friedreich's Ataxia

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Friedreich's ataxia (FA) is a life threatening autosomal recessive disorder for which there is no effective therapy. Most FA patients are homozygous for a GAA trinucleotide expansion in the first intron of the frataxin (FXN) gene, resulting in transcriptional silencing and reduced FXN mRNA and protein levels. While heterozygotes are normal, homozygotes develop neurological dysfunction from 5 to 15 years of age and cardiac dysfunction at 20 to 25 years of age. Two thirds of FA patients die from cardiac dysfunction at an average age of 37.5 years. Average cardiac frataxin levels in FA homozygotes are estimated to be ~9.4 ng/mg protein and heterozygotes ~32.5 ng/mg compared to normal ~59 ng/mg. This provides the target level for vector-derived frataxin expression mediated by gene therapy using intravenous administration of AAVrh.10hFXN, a rh.10 capsid expressing the normal human FXN coding sequence driven by the CAG ubiquitous promoter. We assessed AAVrh,10-FXN function in the MCK mouse model, a severe disease model which has Cre-mediated knockout of the frataxin gene mediated by the MCK promoter confined to cardiac and skeletal muscle. A dose of 1.8 x 10^{12} gc/kg resulted in cardiac FXN levels in MCK mice of 33.7 ng/mg, equivalent to the FXN level in FA heterozygotes. Assessment of mice at this dose resulted in a modest 21.5% improvement in survival from 72 to >88 days ($p<0.01$ compared to PBS controls), with echocardiogram parameters lower than those of wildtype mice ($p<0.001$). To determine if there was additional benefit from higher doses and higher frataxin expression levels, we administered to 7-week-old MCK mice doses of AAVrh.10FXN increasing by half-log increments of 5.7 x 10^{12}, 1.8 x 10^{13} and 5.7 x 10^{13} gc/kg. With the reference of the original dose (1.8 x 10^{12} gc/kg) based on heterozygous cardiac FXN levels as the target, we refer to these as 3.3X, 10X and 33X dose cohorts. Following vector administration, health and behavioral assessments, echocardiograms, and cardiac and liver human FXN levels were obtained. All of the higher dose cohorts had human cardiac FXN levels in excess of the target based on non-FA human heart rising to 20,800 ± 3,200 ng/mg protein in the 33X dose cohort. There was an increase in survival increasing from a median of 87.5 days at the reference dose (1.8 x 10^{12} gc/kg) to 119 days in the 3.3X cohort, plateauing at 128 days in the 10X cohort. At the 33X dose, there was a varied impact on survival, with some animals surviving over 200 days but with some deaths earlier than those in the 10X cohort, likely representing toxicity at this very high dose. Assessed by echocardiogram, compared to the untreated control mice, there was a significant, dose-dependent increase in ejection fraction at 11 weeks from 18.9 ± 4.5% to 31.9 ± 2.9% in the reference dose cohort, increasing to 54.5 ± 9.8% in the 10X dose cohort. At the 33X dose, there was a decrease in ejection fraction, consistent with cardiac toxicity. In summary, there is a substantial survival and cardiac benefit in AAV-mediated expression of frataxin above the normal physiological level achieved at the 10X reference dose in the MCK mouse model of Friedrich's ataxia, but toxicity at high doses. This provides a rationale for ascending the dose in human clinical studies, but with caution regarding toxicity.

364 Development of AAV-Expressed C5 Inhibitor to Locally Suppress Complement Pathway Activation in the Eye as a Potential Treatment for Dry Age-Related Macular Degeneration

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REGENXBIO Inc., Rockville, MD

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population. Multiple lines of evidence suggest that dysregulation of the complement pathway plays an important role in AMD pathophysiology. Results from recent clinical trials demonstrate that monthly intravitreal injections with biologics that suppress complement pathway activation significantly delay lesion growth and disease progression in patients with geographic atrophy (GA), the dry form of late-stage AMD. Despite these encouraging results, monthly eye injections for the duration of a patient's life impose considerable treatment burdens. A single treatment with a gene therapy expressing complement pathway inhibitors in the eye represents an alternative approach that could both reduce the treatment burden and

deliver therapeutic molecules for localized inhibition. Here we develop multiple AAV-expressed inhibitors that target human or mouse C5. We evaluated expression and potency using *in vitro* assays and *in vivo* murine models. We generated AAV constructs expressing C5 inhibitors in multiple formats, including anti-human C5 IgG (150 kDa), Fab (50 kDa), scFV (25 kDa), anti-mouse C5 IgG (150 kDa), Fab (50 kDa), scFV (25 kDa). A tick C5 inhibitor (17 kDa) was also tested in parallel. Media collected from transfected HEK293 cells were examined for transgene expression and for suppression of complement pathway activation in hemolysis inhibition assays. In addition, we generated recombinant purified proteins of each C5 inhibitor to compare their IC_{50} values in the classical and alternative complement pathways using hemolysis inhibition assays. The binding kinetics and affinity of each C5 inhibitor to human, cynomolgous macaque, and mouse C5 were measured with the Octet^{RED}384 system. All three formats of human C5 inhibitor demonstrated K_D values for human C5 in the low to high picomolar range, whereas the tick C5 inhibitor bound less strongly with a low nanomolar affinity constant. We next developed an *in vitro* model of complement overactivation by inducing the formation of the end-product of the complement pathway, membrane attack complex (MAC) on ARPE and iPSC-RPE cells, which are a target for complement deposition in AMD. Treating the cells with purified or vectorized C5 inhibitors prevented C5 cleavage and reduced MAC formation on the cell surface. The anti-C5 factors potently suppressed MAC formation in both systems tested with IC_{50} values in the nanomolar range. The ability of the C5 inhibitors to block C5 cleavage into C5a was also examined in media from the ARPE and iPSC-RPE cells. Dose-dependent reductions in C5a generation were observed with multiple formats of C5 inhibitors. AAV8 encoding C5 inhibitors were injected into wild-type mouse eyes via subretinal administration to determine the distribution, level, and bioactivity of the transgene products (TP) in the mouse eyes. All TPs tested showed high levels of expression and bioactivity. Collectively, our data support the potential of vectorized C5 inhibitors as a treatment for dry AMD.

365 Comparison of *In Vitro* and *In Vivo* Potency of AAV8 Constructs to Correct RDH12 Deficiency

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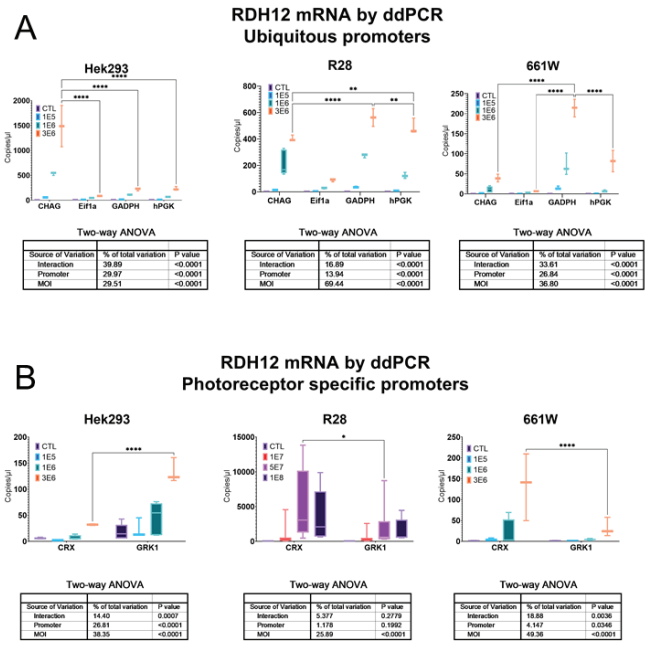
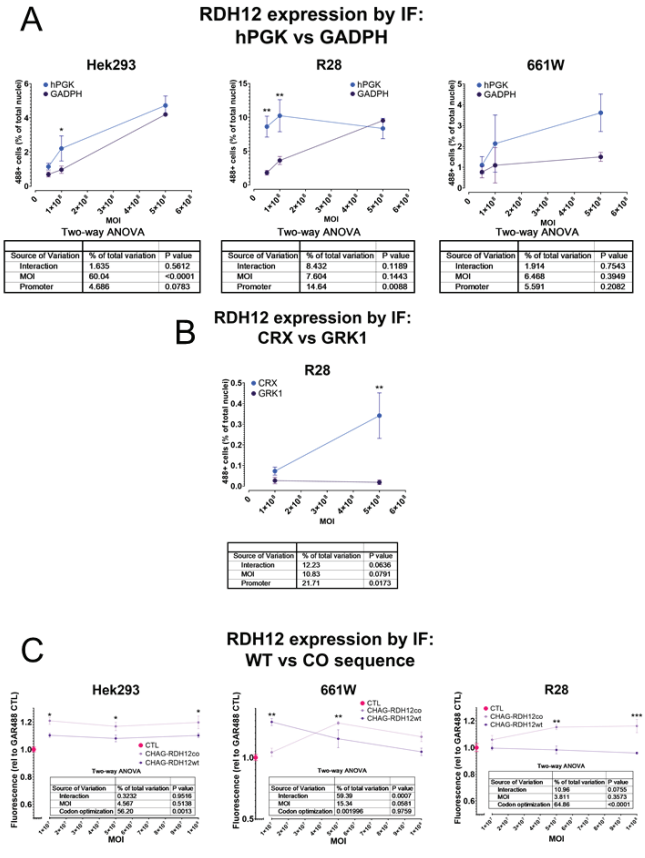
Inherited retinal degeneration may lead to blindness and is caused by mutations in over 200 genes. One of them, *RDH12*, codes for an enzyme involved in the visual cycle; its deficiency leads to early onset blindness in humans. In preclinical studies, RDH12 deficiency causes a susceptibility to light damage in mice. There have been some published successful efforts in producing AAV gene therapies capable of restoring RDH12 expression; however, these did not produce a phenotype that showed morphological changes in the retina or correction of the phenotype using a murine version of RDH12. We aimed to find an AAV construct to express human RDH12 in RDH12 KO mice and protect them from light induced degeneration. To achieve this, we compared the strengths of different ubiquitous and photoreceptor-specific promoters (hPGK, GADPH, Eif1a, CHAG, GRK1, CRX) driving a wild type or a codon optimized RDH12 sequence in vitro and in vivo. Data was analyzed with Graphpad Prism using 2 way - ANOVA tests and the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli for post-hoc comparisons. To compare promoter potency, human (HEK293) and murine retinal progenitor (R28 and 661W) cell lines were transduced with AAV8 with the different constructs at multiplicity of infection (MOI) 1 x 10^5 to 5 x 10^8 virus particles per cell; ddPCR and immunofluorescence were performed to quantify RDH12 mRNA and protein expression, respectively. Considering results from Hek293, R28 and 661W cells, CRX and hPGK were selected as the best performing photoreceptor-specific and ubiquitous promoter, respectively (Fig. 1 & Fig. 2A, B; most significant comparisons and MOI ranges shown). To select between the codon optimized and the wild type sequence of RDH12, the same cell lines were transduced with AAV8 with CHAG as promoter at MOI 1 x 10^7 to 1 x 10^8 virus particles per cell. The codon optimized sequence produced more fluorescence in all cell lines (Fig 2C; most significant comparisons and MOI ranges shown). Finally, to select between hPGK and CRX, we will compare their effects in vivo. RDH12 KO C57BL/6N mice will be injected subretinally with AAV8 carrying the codon optimized RDH12 under CRX or hPGK promoters at doses ranging from 1E8 to 1E10. RDH12 mRNA will be quantified by qPCR. RDH12 protein will be quantified in photoreceptor cells by immunofluorescence and flow cytometry. These results are pending. In a follow up study, the best performing construct will be tested as a potential therapeutic for light induced retinal degeneration in C57BL/6N RDH12 KO mice.



Post hoc comparisons: *P<0.05; **P<0.01; ****P<0.0001



Post hoc comparisons: *P<0.05; **P<0.01

366 *In Vivo* Neural Regeneration via AAV-NeuroD1 Gene Delivery to Astrocytes in Neonatal Hypoxic Ischemic Brain Injury

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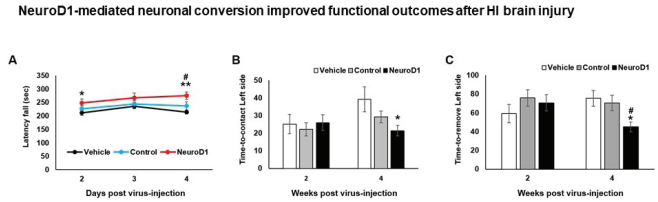
Neonatal hypoxic-ischemic (HI) brain injury is a major cause of massive neuronal loss and neonatal mortality and long-term neurodevelopmental disability. Current therapeutic approaches for HI brain injury have been limited to general supportive therapy because of the lack of methods to compensate irreversible neuronal loss. Recently, i*n vivo* direct neuronal reprogramming technology, which can avoid exogenous cell supply and immune suppression, has emerged as a powerful approach to compensate for the loss of neurons. We report an neuroregenerative therapy through *in vivo* transdifferentiation of astrocytes to neurons based on AAV-NeuroD1 gene delivery in neonatal HI brain injury mouse models, that have shown anatomical and functional recovery. The AAVShH19 vector, which has a higher rate of gene transfer to astrocytes than parent AAV2, and the a Cre-FLEx system for astrocyte-specific and long-term tracking of infected cells were used. AAVShH19-mediated ectopic expression of NeuroD1 in an HI brain injured mouse model converted astrocytes into GABAergic neurons and the converted cells had electrophysiological properties and synaptic transmitter. Additionally, we found that NeuroD1-mediated *in vivo* direct neuronal reprogramming protected injured host neurons and altered the host environment, represented as reduced number of activated microglia and reactive astrocytes, reduction of toxic A1-type astrocytes, and reduced expression of proinflammatory factors. Furthermore, NeuroD1-treated mice significantly improved their motor functions. Together, this study demonstrates that NeuroD1-mediated *in vivo* direct neuronal reprogramming technology through AAV gene delivery as a novel regenerative therapy for neonatal HI brain injury.



367 Translational Volumetric Capacity of the Cerebrospinal Fluid Compartment between Humans *and* Nonhuman Primates

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The cerebrospinal fluid (CSF) plays a crucial role in the homeostasis of the central nervous system (CNS) and has also been known to provide protection from exogenous trauma. Exploration of different routes of administration (RoAs) as potential avenues for gene therapy delivery have led to the reaffirmation that adeno-associated viral (AAV) vectors can indeed infect nervous tissue following CSF administration. Using magnetic resonance (MR) images acquired on a 3T scanner, donor scans from adult cynomolgus (N=10), pediatric human (N=10) and adult human cases (N=10), were analyzed using ClearPoint's Navigational Software, ITK-SNAP and OsiriX for corresponding volumetric values for CSF compartments. Interestingly, volumetric analysis of the cisterna magna lumen revealed a clinical variation of 7-fold in adult humans, versus a 5-fold variation in both pediatric humans and in nonhuman primates (NHPs). Comparatively, the difference in the averaged volume of the cisterna magna between human adults versus NHPs was 10-fold, whereas it was 9-fold between pediatric MRIs and those of NHPs. Examination of fourth ventricular lumens evinced a lower intra-subject variation clinically, as opposed to a greater inter-species variability between NHPs and humans. The volumetric data, in addition to measurements collected along a 3-dimensional axis, provide important translational considerations when designing *in vivo* animal studies and clinical trials aimed at delivering gene products to the cisternal and fourth ventricular lumen.

368 Barogenetic Gene Therapy for Modulating Blood Pressure

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Introduction: Cells of different organs in the body undergoes exposure to range of pressures which changes in various diseases including neurological, cardiovascular, ophthalmological, and renal diseases. Clogging of arteries leads to hypertension and related injury. In case of hypertension, blood exerts pressure against the walls of blood vessels that can lead to severe health complications and increase the risk of heart disease, stroke, and sometimes death. We have engineered a mechanosensitive channel from bacteria that acts as pressure sensing and actuating molecule (SAM) and shown that heterologously expression of SAM in targeted cells leads to reduction of hypertension. **Methods and Mechanism of Action:** To investigate the effect of SAM-gene therapy for reduction of blood pressure, mouse model of hypertension (BPH/2J, Jackson lab) was used. The mice were injected with 175 µl of rAAV-SAM in tail vein. After 2 weeks of injection, tail cuff blood pressure measurement via Volume Pressure Recording (Kent Scientific) was carried out. Blood pressure was also recorded for non-injected BPH/2J mice (positive control) and wildtype mice. After these measurements, the mice were sacrificed, and tissues from various vital organs were collected. Immunohistology was performed on tissue sections to visualize the reporter (mCherry) expression. SAM expression in the endothelial cells of the glomerulus or the excretion apparatus in the kidney is expected to sequester fluid from the vasculature into the interstitial spaces or the glomerular space for later excretion from the body. Auto-regulating activity of the SAM molecule may induce an overall volume loss from the systemic vasculature, leading to a decrease in overall blood pressure.**Results:** Systemic injection of rAAV-SAM led to robust expression in the endothelial cells of the glomerulus in kidney. SAM expression in hypertension mouse model led to significant reduction of elevated Systolic and diastolic blood pressure to a level similar to that of the wildtype mice. Thus, SAM therapy demonstrated to have the same end physiological effect as diuretics but with the added benefit of self-regulation and permanence (as it would activate as soon as elevated blood pressure induced tension in the blood vessels reaches a pre-determined threshold). **Conclusions:** The engineered sensing and actuating molecule (SAM) is activated at physiological pressures, thereby enabling significant reduction of blood pressure in hypertension mice model. The SAM-gene therapy approach has potential to be transformative in maintaining health and physiological functions of various organs in a gene-agnostic manner for chronic diseases involving hypertension.

370 Exploring Gene Addition of a Single Unit within the Multi-Subunit Enzyme Complex of Serine Palmitoyl Transferase

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Sphingolipids (SLs) are lipid molecules that play essential structural and signaling functions in eukaryotes and have been linked to many neurological diseases. Serine palmitoyl transferase complex (SPT) mediates the first and rate-limiting step in the de novo sphingolipid biosynthetic pathway. The larger subunits SPTLC1 and SPTLC2/SPTLC3 together form the catalytic core with a smaller third subunit either SSSPTA or SSSPTB propelling the catalytic efficiency and providing substrate specificity for the fatty acyl-CoA substrates. Recently, the SPTSSA^{T51I} mutation was discovered in two girls with stagnant neurodevelopment, and both showed substantially elevated levels of several SLs. To further investigate the pathophysiology of this disease-causing mutation and explore therapeutic approaches, we developed a mouse model T51I knock-in mouse using targeted introduction of the T51I allele into exon 2 of the *SPTSSA* gene by CRISPR technology in fertilized eggs and further assessed the sphingolipid biochemistry and behavior in these mice. While the Sptssa^{T51I} heterozygous mice had minimal neurological deficits (sensory, motor or cognition) up to 5 month of age, biochemical analysis revealed an elevation in SL levels. Surprisingly, supplementation with 10% L-serine for 2-3 months further increased SL levels and now worsened the hind limb clasping behavior, suggesting a critical threshold in lipid chemistry impacting motor behavior. *In vitro* data in patient fibroblasts demonstrated successful transduction with adeno-associated virus (AAV) vector encoding wildtype SPTSSA (AAV-SPTSSA) and improvement in lipid chemistry. This has provided the justification for ongoing *in vivo* studies of AAV-SPTSSA gene therapy in Sptssa^{T51I} heterozygous mice. In conclusion, our studies demonstrate the feasibility of adding a single unit of a multisubunit enzyme complex to confer functional benefit. Our preliminary studies in mouse suggest the functional importance of the small subunit variant in Sptssa^{T51I} heterozygous mice by demonstrating the association of hind limb clasping with SL elevations. AAV-mediated delivery in human fibroblasts suggests that addition of SPTSSA can combine with other native subunits and impart its specific catalytic efficiency and restore the normal homeostasis of SPT. This may warrant further *in vivo* therapeutic development in mice and humans.

371 Prosaposin Gene Therapy Improves Features of Gaucher Disease in Preclinical Studies but Does Not Reverse the Neuropathology in Mouse Models of Alzheimer's Disease and Amyotrophic Lateral Sclerosis

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Prosaposin is a highly conserved glycoprotein and the precursor for four lysosomal activator proteins called saposins. These saposins have distinct roles in regulating lysosomal hydrolysis of sphingolipids. Dysfunction of saposins results in an assortment of lysosomal storage disorders (LSD), such as Gaucher Disease (GD) with severe neuropathology. Mutations in the *PSAP* gene can cause a deficiency of either full-length prosaposin or any of the individual saposins, resulting in the accumulation of nondegradable, toxic glycolipids. Secreted full-length prosaposin has been shown to exert neuroprotective and glioprotective effects in cell culture systems but its in vivo application has not been examined. We hypothesized that increasing prosaposin levels might be useful therapeutically for LSDs and neurodegenerative disorders. Using a gene therapy approach, we administered AAV-hPSAP to two established mouse models of GD, the CBE and 4L/PS-NA mouse models via intracerebroventricular (ICV) injections. AAV-hPSAP improved neuroinflammation, and glycolipid levels in the brain with a trend towards improved motor function. To examine the neuroprotective role of prosaposin, we used two mouse models of Alzheimer's disease (AD), APP-PS1 and rTg4510 and a mouse model of Amyotrophic Lateral Sclerosis, TAR6/6. ICV delivery of AAV-hPSAP had no effect on memory dysfunction, phospho-Tau or neurofilament light chain (Nf-L) levels in AD mouse models. In the TAR6/6 model, ICV delivery of AAV-hPSAP did not improve motor dysfunction, Nf-L or TDP-43 levels. In summary, we demonstrate the therapeutic potential of prosaposin in LSD but further work is warranted to understand whether the protective effect of prosaposin can be extended to other neurodegenerative disorders.

372 Antibody Gene Therapy for Rabies Encephalitis

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Introduction. Rabies virus is one of the most crippling neurotrophic viruses with almost 100% fatality rate worldwide. Vaccination strategies that are currently used to prevent rabies infection primarily rely on the generation of the host serum neutralizing antibodies against rabies virus. The major caveat of serum neutralizing antibodies-based therapy is that while the rabies virus can easily breach blood-brain barrier, the antibodies cannot, making them largely ineffective in the treatment of symptomatic brain encephalitis. There is a need for antibody-induced protection in the central nervous system, independent of the host immune system. We propose to use the recombinant AAV9 (rAAV9) viral vector to traverse the blood-brain barrier and express a neutralizing human immunoglobulin against rabies glycoprotein in the brain. **Methods**. rAAV9 vector expressing a broadly neutralizing human antibody against rabies (CR57) was used to treat C57BL/6J mice intravenously for dose escalation study ($1x10^{10}$ to $1x10^{13}$ vg/kg) and longitudinal study ($6x10^{13}$ vg/kg dose and monitored for 60 days). We analyzed the expression of human CR57 in blood samples and brain tissue by Rapid Fluorescent Focus Inhibition Test (RFFIT), ELISA, and IHC. Additionally, 4-month-old cats were treated with low ($2x10^{12}$ vg/kg) or high ($1x10^{13}$ vg/kg) doses of rAAV9 intravenously and tissues were further analyzed. **Results.** The rabies neutralizing antibody level in mouse serum was >200 times higher than the protective titer suggested by the World Health Organization (WHO; 0.5 IU/ml) 1-3 months after the administration of $1x10^{12}$, $1x10^{13}$ and $6x10^{13}$ vg/kg rAAV9 vector. The expression of rabies neutralizing antibodies was further confirmed in neurons and brain endothelial cells by IHC. The level of CR57 was persistent in mouse serum for 60 days post-injection. However, neutralizing antibodies were absent in the lowest dose treatment group ($1x10^{10}$vg/kg). In rAAV9-treated cats, the high-dose treatment group showed significant levels of rabies neutralizing antibodies in serum for at least 15 months and in CSF for 3 months post-injection. Antibodies were also observed in cat serum of the low-dose cohort for 15 months after treatment. The decline in rabies neutralizing antibodies in feline serum of both high and low-dose treatment groups after 15-months is attributed to the development of cat immune response against human CR57. Nonetheless, rabies neutralizing antibodies were detected in the feline brain of the high-dose treatment group at endpoint (25-months post-treatment) by IHC.

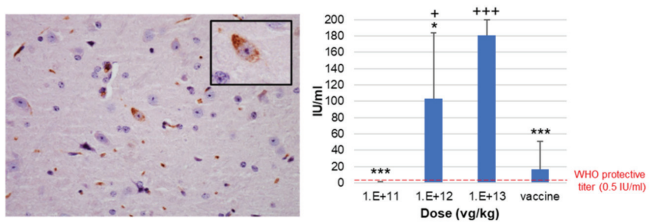

*Fig. Left: Antibody in mouse brain after IV injection of rAAV9 vector (brown staining). Insert represents the magnified neuron. Right: Recombinant AAV9 doses produced rabies neutralizing antibody >200 times above the protective titer (WHO) in mouse serum. ***p<0.0001 or *p<0.05 v. 1e13; +++p<0.0001 or +p<0.05 v. standard rabies vaccine*

Conclusions. An AAV9 vector injected intravenously in two species expresses broadly neutralizing antibodies in brain cells and thereby may provide protection against, or treatment of, viral encephalitis.

373 Development of a Gene Therapy for Alzheimer Disease by Lowering Toxic APOE While Simultaneously Overexpressing a Protective APOE Variant

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Alzheimer Disease (AD) is the most common form of dementia in people over 65 years (~50 million), manifesting as progressive memory loss and decline in cognitive function. The strongest genetic risk factor for late onset AD (LOAD) is the Apolipoprotein E4 (APOE4) variant. This lipoprotein is expressed in the liver and brain, facilitating lipid transport and homeostasis. APOE3 is the most common variant in the population, followed by APOE4 and APOE2 (79>13.3>7.3%). APOE4 is present in 45-60% of all AD cases and is associated with increased risk and decreased age of onset, while APOE2 is deemed neuroprotective. APOE4 depletion in humanized (h) APOE-AD mouse models prevented neurodegeneration in previous studies. We are developing an AAV gene therapy approach to treat patients with LOAD by silencing toxic APOE4 and overexpressing a protective APOE variant simultaneously. To identify the best candidates, a number of APOE silencing miQURE® molecules and protective APOE-variants were first screened in vitro and in vivo separately. We previously demonstrated that microRNAs against APOE using our silencing miQURE platform effectively suppressed APOE expression *in vitro*. APOE-targeting miQURE molecules were tested by intrastriatal delivery of AAV vectors in a *hAPOE4*-transgenic mouse model. Expression of miQURE molecules resulted in potent reduction of hAPOE4 mRNA and protein in the brain. Additionally, various protective APOE-variants were screened *in vitro* for transgene expression and secretion. The best protective APOE-variants were examined *in vivo* by intrastriatal AAV delivery in a human Tau overexpressing transgenic model; P301S Tau-transgenic mice. The expression of the protective APOE-variants and the impacts on pathological phenotypes will be analyzed on post-mortem material seven months post-injection. Finally, the most potent miQURE molecules and protective APOE-variants were selected and implemented into a combined construct to function in concert. The combined constructs that simultaneously encode miQURE and protective APOE-variant transcripts were validated in an *in vitro* screen to demonstrate miQURE efficacy as well as APOE-variant expression. A subset of these constructs was selected for *in vivo* testing via intrastriatal delivery of AAV vectors, whereafter the expression of the transcripts will be examined. In conclusion, simultaneous silencing of toxic APOE and overexpressing a protective APOE variant warrants further investigation as a gene therapy approach for AD. *miQURE is a registered trademark in the US and other jurisdictions.*

374 An Adeno-Associated Virus-Based Combination Gene Replacement Therapy for Parkinson's Disease

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Parkinson's disease (PD) has an incidence rate as high as 1%-2% among people over 60 years old, and is the second largest neurodegenerative disease in the world. In China, it is estimated that there are more than 2 million PD patients. The pathogenesis of PD is hypothesized to be the combined effects of gene mutations and the environmental factors. The main pathological changes of PD are the loss of dopaminergic neurons in the substantia nigra, and the appearance of Lewy bodies (mainly the aggregation of α-synuclein protein) in the cytoplasm of the remaining neurons, leading to the irreversible neurodegeneration of dopaminergic projections from the substantia nigra to the striatum. With the reduction of dopamine in the striatum, typical motor symptoms (Parkinsonism) appear, including resting tremor, muscle rigidity, slow movement, posture and gait disorders, and when degeneration affects other brain regions, obvious non-motor symptoms such as sleep disturbance, depression, cognitive impairment, *etc*., will also appear. The currently available treatments are mainly to increase dopamine levels in the brain by providing dopamine derivatives, like levodopa. These treatments have limited efficacy for non-dopaminergic symptoms, especially in advanced stage of PD and are unable to change the disease progression. Moreover, as the disease progresses, the side effects, for example escalating dose of levodopa induced dyskinesia, gradually become intolerant. New therapies that could not only effectively improve motor symptoms, but also slow the disease progression and delay the onset of disability of other brain functions are urgently needed for PD patients. In the normal brain, dopamine is converted from tyrosine to dopa catalyzed by tyrosine hydroxylase (TH), and then into dopamine by dopa decarboxylase (AADC). The progressive decrease of AADC expression in the striatum of advanced PD patients has been hypothesized to be one of the reasons for reduced responses to levodopa. Therefore, supplementing AADC in the striatum could effectively increase the conversion efficiency of levodopa into dopamine and reduce the side effects induced by levodopa overdose. Meanwhile, to stop the progressive degenerative changes in patient's brain, neuroprotective intervention like overexpressing neurotrophic factors has shown to be a promising strategy. Based on the above hypothesis and our knowledge of PD pathologies, we have developed a combination gene therapy to address simultaneously the different aspects of PD pathologies to provide a disease modifying long-term

treatment option for PD patients. In the current study, VGN-R09b, as a recombinant AAV9, delivers genetically modified human AADC and a neurotropic factor genes, which are directly distributed in the striatum, to infect nerve cells through brain parenchymal injection. The AADC delivered by VGN-R09b will increase dopamine levels in the synaptic cleft to improve motor symptoms. The neurotropic factor delivered will be secreted extracellularly, act on the synaptic terminal of dopaminergic neurons to promote axonal sprouting and produce neuroprotection effects. In preclinical mouse studies, intra-striatal injected VGN-R09b appeared to be well-tolerated at doses up to 2×10^{12} vg/kg. Intra-striatal injection of VGN-R09b could dose-dependently improve the motor impairments, increase the striatal AADC activity, and protect the dopaminergic terminals in PD rodent and monkey models. Our preclinical data demonstrated the safety and efficacy of VGN-R09b and indicated its potential as an effective long-term treatment for PD.

375 A New Minimally Invasive Endovascular Approach to the Cerebello-Pontine Cistern Enables Improved AAV Biodistribution Compared to Cisterna Magna Injection

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Introduction: Delivery of viral vectors to target brain tissue remains an unsolved challenge due to the blood brain barrier. Parenchymal brain injections or cerebrospinal fluid delivery to the cisterna magna (CM) can improve CNS delivery, but with unfavorable risk profile. A novel approach that leverages advances in catheter intervention, imaging technology, and operator workforce could improve safety and efficacy, and resolve the challenge of delivering clinically useful gene and cell-based therapies to the brain. We have previously presented a percutaneous endovascular CNS access system to the cerebello-pontine angle (CPA) cistern located in front of the brainstem. Considering the characteristics and limitations of current techniques, here we determine how biodistribution of scAAV9-CB-GFP administered into the CPA compares to that of the same vector into the CM. **Methods:** Eight sheep were allocated into CPA (n=4) and CM (n=4) cohorts. Lumbar puncture was performed into the lumbosacral intervertebral space (L7-S1). A 1.7F braided microcatheter with 0.014" micro-guidewire was introduced into the subarachnoid space and navigated into either the CPA cistern or CM under fluoroscopic guidance. Cone-beam computed tomography fused with MR imaging was used to confirm accurate final placement of the catheter. scAAV9-CB-GFP (1.0×10^{14} vg in 3mL) was injected at 200µl/min. Animals were sacrificed 3 weeks post-procedure. Anti-GFP antibody immunohistochemistry was performed with (Abcam ab290). Vector genome biodistribution was determined by qPCR using primers and probes for the BGH poly (A). **Results:** Immunohistochemistry targeting GFP shows a stronger transduction profile in the parietal cortex in the CPA cohort compared to CM (Fig 1). In the dorsal midbrain, a modest increase of GFP immunoreactivity was observed in the CPA cohort. Strong immunoreactivity was also observed in the ventral aspect of the cingulate gyrus. Vector genome quantification of the occipital cortex and midbrain tissues revealed comparable vector genomes between CPA and CM injection routes at approximately 10,000 vg/100ng (Fig. 2). Only modest biodistribution to peripheral liver tissue was observed with both injection routes. **Conclusions:** CPA delivery of AAV9 resulted in increased transduction of the parietal and cingulate cortex, compared to that observed with CM injections. These results suggest that the clinically safer percutaneous endovascular minimally invasive method of delivery to the CPA cistern may offer a more effective approach than cisterna magna injection for diffuse delivery of gene and cell-based therapies to the CNS.

Figure 1: GFP expression 3 weeks following Cerebellopontine Angle Cistern (***left***) and Cisterna Magna (***right***) injection of scAAV9-CB-GFP.

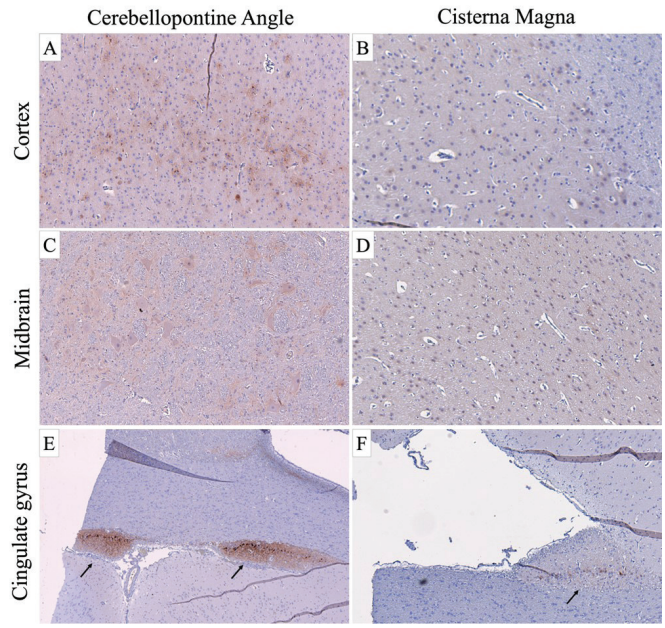
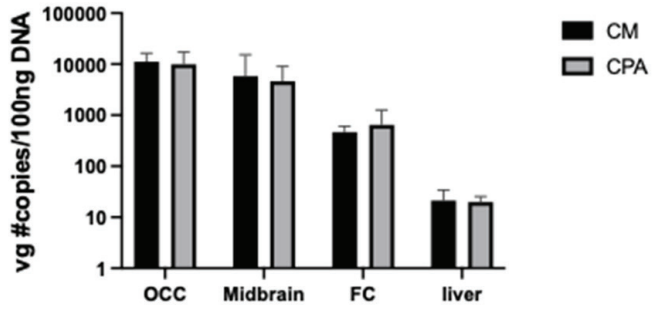


Figure 2: AAV biodistribution to occipital cortex (OCC), midbrain, frontal cortex (FC) and liver.



376 Ocular Biodistribution of AAVHSCs across Species and Routes of Administration

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A current challenge for ocular gene therapy is choosing an appropriate delivery vehicle and route of administration to target diseased cells precisely and robustly. Although several options of dosing routes have emerged, cell-type selective delivery vehicles are limited. Adeno-associated viruses (AAVs) show promise for gene therapy in the eye due to their stable transgene expression in non-dividing cells. We have reported the discovery of 15 novel, naturally occurring, Clade F AAVs derived from human hematopoietic stem cells (HSCs). Here, we present the complete ocular biodistribution of 11 AAVHSCs to demonstrate their utility as delivery vehicles for a variety of indications. We evaluated tropism of each AAVHSC capsid for individual ocular cell types by packaging one of two transgenes: single-stranded enhanced green fluorescent protein (ss-eGFP) transgene driven by the CMV promoter or self-complementary enhanced green fluorescent protein (sc-eGFP) driven by the chicken beta actin (CBA) promoter. We first targeted the posterior segment of the mouse (C57Bl6/J) eye by three dosing routes, subretinal, intravitreal (IVIT) and suprachoroidal. We then extended subretinal dosing to two larger mammals, non-human primates (NHPs, cynomolgus macaque) and Gottingen pigs. To target the anterior segment of the mouse eye, we dosed each capsid by one of two routes, IVIT or intracameral. Finally, we dosed human retinal explants with ss.AAVHSC15.eGFP. In all species, biodistribution of each AAVHSC was assessed by multiplexed immunostaining with anti-eGFP along with cell-type specific markers. Key observations include: 1) Across all species tested, including in human cells and in NHPs, AAVHSCs transduced photoreceptors (PRs) and retinal pigment epithelial (RPE) cells, both targets for inherited retinal disease; 2) A subset of AAVHSCs transduced murine PRs and RPE by the preferred IVIT dosing route; 3) Other murine retinal cells of therapeutic interest, Müller glia and retinal ganglion cells, were also transduced in a capsid-selective manner; 4) AAVHSCs exhibited capsid-specific tropism for trabecular meshwork cells, a cell type key to the onset of glaucoma; 5) Specific AAVHSCs exhibited the ability to cross the blood-retina barrier following a suprachoroidal dose, the least invasive ocular route of administration. These data demonstrated that AAVHSCs transduced several clinically relevant cell types across multiple species, allowing potential for gene therapy application in treating ocular diseases affecting both the front and back of the eye.

377 Development of an AAV-Based Gene Therapy for Dermatosparaxis Ehlers-Danlos Syndrome

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Introduction Dermatosparaxis Ehlers-Danlos syndrome (dEDS) is an autosomal recessive connective tissue disorder caused by a loss of function mutation in ADAMTS2 encoding for procollagen I N-proteinase. The mutation impairs the cleavage of the N-propeptide from type I procollagen chains resulting in mature collagen fibrils with aberrant morphology and lower tensile strength. Patients with dEDS present with severe skin fragility and redundant skin, hernias, craniofacial deformities, short stature, easy bleeding and bruising, visceral fragility, and premature placental rupture of the membranes. This type of EDS was selected for development of a gene therapy, because 1) it is caused by a loss of function of a secreted protein (ADAMTS2), 2) it is encoded by a 3.6kb cDNA, and 3) ADAMTS2 is ubiquitously expressed throughout the body. Due to the extracellular action of ADAMTS2, AAV9 was selected with the goal of transducing liver and skeletal muscle to provide stable long-term expression. The knockout mouse model of ADAMTS2 (*Adamts2*^{KO}) also develops skin fragility, loss of surface contour of the molar teeth, abnormal lungs with a pseudo-emphysematous appearance, and sterility of male mice.
Methods Four AAV9 vectors encoding for various elements to optimize expression and secretion were screened for toxicity in 6-week-old C57BL/6J mice. Mice in each group (n=4) were treated via intravenous tail vein injection at a dose of 1E12 vg/mouse. Mice were sacrificed one month later. Subsequently, *Adamts2*^{KO} (n=6) and normal littermate controls (n=6) at 1E12 vg/mouse were injected via intravenous tail vein injection post-symptomatically at 2 months of age. Two endpoints, 2 months and 1 year, will be employed for each group.
Results The wild type mouse study produced no adverse clinical outcomes in any mice, and tissues appeared grossly normal at necropsy. Histology, transmission electron microscopy, qPCR, and western blot are underway. *Adamts2*^{KO} mice will be evaluated based on clinical signs, skin fragilty, necropsy findings, transmission electron microscopy, biochemical electrophoretic analysis of procollagens, qPCR analysis of ADAMTS2 expression and vector genomes to select the best vector. Following this, a clinical trial will be initiated using a naturally occurring dog model of dEDS.

378 Significance of Pre-Existing Antibodies to Multiple Serotypes of AAVs in NHPs Serum, Measured by NAb and TAb Assays

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VRL, San Antonio, TX

The presence of pre-existing neutralizing antibodies (NAb) against adeno associated viruses (AAVs) has the potential to negatively impact the transduction efficiency when they are used as delivery vectors in gene therapy studies. With AAV emerging as one of the most efficient viral vectors, it is now more relevant to have effective and consistent tools to measure anti-AAVs antibodies. Cell-based assays to measure NAbs, and ELISA to measure total antibodies (TAbs), are the most commonly used methods to enumerate anti-AAV antibodies. In this work we used both techniques to correlate and determine the significance of having antibodies to several serotypes. We investigated what could be the impact of the presence of multi reactive antibodies measured by TAb-ELISA in their capacity to neutralize a specific AAV serotype. To this end we tested a panel of 171 NHP serum samples. Five different AAV serotypes, AAV1, AAV2, AAV5, AAV8 and AAV9 were used for the analysis. For the NAb assay, the AAV reporter for each serotype, encodes for the green fluorescent protein and was used to measure the percentage of neutralization inhibition. For the TAb assay, virus-like particles of the five serotypes were used to measure total IgG in the serum samples by an indirect ELISA. The implications of multi-reacting samples in the neutralization of the corresponding serotype and other serotypes of AAV will be further discussed.

379 Rational Engineering of Novel Synthetic Promoters for Specific AAV-Delivered GRN Expression in the CNS

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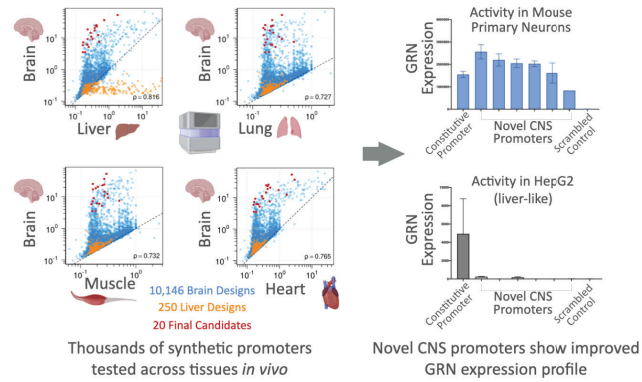
Safe and effective gene therapies using adeno-associated viruses (AAVs) require payload expression at high levels in target cells with minimal expression in undesirable tissues, especially for therapies targeted outside the liver. Promoter sequences are key levers to control gene therapy transgene expression, but most "off-the-shelf" promoters are not sufficiently strong, or tissue-specific, or compact enough for use in AAV-based gene therapy. To address these challenges, we developed a synthetic biology platform that enables discovery of highly specific and tunable promoters that are tailored to a desired target product profile (e.g., central nervous system (CNS-specific). We employ Massively Parallel Reporter Assays (MPRAs) to screen thousands of promoters or promoter components in each experiment. Our approach is based on the modular framework of a highly optimized core promoter combined with a cell-type-sensing enhancer region, whereby the final promoter is of a consistent and small size (~200 bp). Here we demonstrate the development of brain-specific promoters for a Progranulin (GRN) gene replacement transgene for the treatment of frontotemporal dementia (FTD). We aimed to engineer a synthetic promoter that provides robust CNS-specific expression of a GRN transgene with minimal expression in other AAV-transducible tissues such as liver, heart, and skeletal muscle. We first performed a 3,600-candidate MPRA screen that identified an improved core promoter yielding three-fold higher expression than industry-leading standard core promoters like ybTATA and minP and no loss in dynamic range. We then engineered 10,000 candidate CNS-specific enhancers by mining human functional genomic databases and screened by MPRA in both in vitro cell and in vivo mouse models. Final candidates were selected for high expression in CNS models including mouse brain and the human neuronal LUHMES cell line, with low expression in mouse liver, heart, skeletal muscle, kidney, and lung, and a human liver-derived HepG2 cell line. The top performing CNS-enhancers were combined with the core promoter to yield final promoter constructs that displayed improved strength, specificity, and size profiles compared with currently available constitutive or CNS-specific promoters. In addition to identifying CNS-specific, liver-inert promoters, our modified core promoter plus enhancer discovery approach can be adjusted rapidly for other desired tissue expression profiles, as the underlying genomic datasets already exist body-wide. In particular, the combination of specific and tunable promoters with emerging novel tissue-specific AAV capsids promises to accelerate the development of safe and effective gene therapies.



380 Cell Class-Specific Gene Therapy for Dravet Syndrome

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Dravet syndrome (DS) is a devastating developmental and epileptic encephalopathy marked by treatment-resistant seizures, developmental and motor deficits, and a high rate of premature death. In over 80% of patients with DS, the disease is caused by heterozygous loss-of-function mutations in SCN1A, the gene encoding Nav1.1 channels. SCN1A mutations impair GABAergic interneuron excitability, and mouse models recapitulate key DS

phenotypes when SCN1A mutations are targeted to GABAergic interneurons. Taken together these observations suggest interneurons constitute a promising target for precision gene therapy in DS. Here we developed a GABAergic interneuron-targeted AAV SCN1A gene replacement therapy using highly validated cell class-specific enhancers. Using these tools we circumvented AAV carrying capacity limitations and achieved full-length SCN1A expression in forebrain GABAergic neurons with high efficiency and specificity. These constructs produce functional Nav1.1 protein as evidenced by patch-clamp recordings and western blot in cell lines. After packaging these vectors into AAV and administration in mice, immunohistochemical analyses showed dose-dependent biodistribution of the therapeutic cargo in targeted cells and brain regions. Remarkably, these vectors conferred strong dose-dependent protection against mortality and thermally induced seizures to DS mouse models carrying nonsense alleles of Scn1a. We observed protection in two independent model mouse lines and at two independent research sites, with no overt toxicity observed in littermate control mice. Immunohistochemistry on these testing cohorts allowed us to directly correlate vector biodistribution with functional rescue. Furthermore, we also tested additional vectors for pan-neuronal expression of Nav1.1, and these vectors provided weaker protection from DS symptoms, and also showed safety concerns including preweaning lethality. As a whole these findings suggest that cell class specificity might not only boost effectiveness of gene therapy in DS, but could also make therapy safer. In summary these findings demonstrate proof-of-concept that cell class-specific AAV-mediated SCN1A gene replacement therapy could provide an effective precision therapy in DS.

381 ICV Administered AAV9-NGLY1 Gene Replacement Therapy (GS-100) Improves Motor Function and Biomarker Outcomes in Ngly1 Deficient Rats

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GS-100 is an AAV9 gene therapy for the treatment of N-glycanase 1 (NGLY1) Deficiency, an ultra-rare, debilitating recessive disorder due to loss-of-function mutations in the NGLY1 gene. Symptoms include global developmental delay, intellectual disability, hyperkinetic movement disorder, elevated liver enzymes, (hypo)alacrima, and peripheral neuropathy. There are no approved therapies for this disease. Preclinical route of administration and dose-finding studies with GS-100 demonstrated positive treatment effects starting at 6.2e11 vg/animal in Ngly1-/- rats following intracerebroventricular delivery. We evaluated the dose-dependent efficacy and safety of GS-100 in a 6-week pharmacology/toxicity study in Ngly1-/- rats at three dose levels (6.2e11, 6.7e12, and 1.1e13 vg/animal). Widespread dose-dependent biodistribution of GS-100 vector DNA was detected in the brain and spinal cord, which positively correlated with hNGLY1 mRNA and protein expression. Treatment with GS-100 resulted in a dose-dependent improvement in motor function (rotarod) and reduction of the NGLY1 disease biomarker, GNA, in the cerebrospinal fluid (CSF) and brain tissue. The low dose of GS-100 was associated with non-significant trends in rotarod improvement and GNA reduction in the Ngly1 deficient rats, whereas the mid-dose was associated with statistically significant improvement in motor function to near wild-type levels that correlated with a statistically significant reduction in GNA (42.5%). Similar rotarod findings were detected at the high dose, with slightly more reduction in the GNA biomarker (45.9%). GNA levels in the CSF were found to correlate with GNA levels in brain tissue, supporting the use of CSF GNA levels as a surrogate measurement for brain tissue GNA levels. Importantly, an inverse correlation was observed between motor improvements and the GNA biomarker in CSF and brain tissue, supporting the use of CSF GNA as a surrogate marker of clinical endpoint improvement in a clinical setting.GS-100 was well tolerated at all dose levels tested in this study. There were no systemic toxicities, significant increases in liver transaminases, or macroscopic pathology attributed to GS-100. Histopathology analysis revealed dose-dependent, mild to moderate neuronal loss and gliosis in the hippocampal region of the brain near the injection site, at all dose levels. Statistical analysis found no correlation between the severity of the neuropathologic findings and motor performance within individual animals. Correlations between vector biodistribution, hNGLY1 expression, GNA biomarker reduction, and motor function improvement in Ngly1 deficient rats indicate that ICV administration of GS-100 results in the expression of functional hNGLY1 that ameliorates the underlying disease pathology, resulting in positive disease modification.

382 Crossing the Non-Human Primate Blood Brain Barrier with Machine-Guided AAV Capsid Design

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AAV capsids that efficiently cross the blood brain barrier and transduce the brain are necessary to facilitate gene therapy for many neurological disorders but remain a major unmet need. Here, we describe the discovery and characterization of a novel capsid exhibiting substantially improved transduction of the CNS when delivered intravenously in non-human primates (NHPs). In high throughput screens, this capsid consistently demonstrated large improvements in transduction efficiency relative to AAV9, including an 80-fold improvement in African green monkeys and a 100-fold improvement in cynomolgus macaques. Moreover, in an NHP validation study at modest dose (1e13 vg/kg), histology analysis showed that the selected capsid transduced >10% of all cells, and up to 25% of neurons in therapeutically relevant brain regions including the hippocampus, basal ganglia, cerebellar cortex, and substantia nigra. The capsid exhibited 5-fold reduced biodistribution and 10-fold reduced transduction in the NHP liver relative to AAV9, and produced comparably to AAV9. The discovery of this capsid leveraged machine-guided design to identify an AAV capsid sequence with greatly improved CNS transduction properties over AAV9 as well as additional properties of therapeutic interest. The sequence changes include a novel motif relative to all known engineered capsids that is not a peptide insertion. Iterated application of machine-guided design has identified further-improved capsids that warrant additional characterization given the high concordance between our discovery and validation studies demonstrated here.

383 A Multiplexed Barcode Approach to Simultaneously Evaluate Gene Delivery by AAV Capsid Variants Across Multiple Non-Human Primate Tissues

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Adeno-associated virus (AAV) vectors have been widely used to deliver therapeutic transgenes to many distinct cell populations or tissues across countless studies. Both naturally occurring and engineered AAV capsids have been independently investigated as AAV vectors, and extensive studies have been performed in mice and other species to determine their *in vivo* tropisms. However, studies in mice are not always predictive of vector performance in other animal models or humans, and differences in tissue tropisms have been observed between different mouse strains. Considering the increasing interest in AAV vectors for human gene therapy, an increasing number of studies are being performed in large animals such as non-human primates (NHP), yet studies in large animals have also shown that significant species-to-species, and often within-species variation exists. To optimize the process of AAV capsid selection and keep animal numbers to a minimum, we developed a multiplex barcoding approach that allows the simultaneous evaluation of *in vivo* vector performance for multiple different capsids. We assessed vector biodistribution and gene transfer in male and female rhesus macaques that were simultaneously administered a combination of 8 or 11 naturally occurring and capsid engineered barcoded AAV vectors intravenously. Animals were immune suppressed with rapamycin and tacrolimus for 2 weeks prior to AAV administration and for the duration of the experiment to suppress pre-existing anti-AAV immune responses, and then followed longitudinally post AAV administration to determine which AAV capsids were able to successfully transduce different tissues using a combination of qPCR, qRT-PCR, vector DNA Illumina amplicon sequencing (vDNAseq), and vector mRNA Illumina amplicon sequencing (vRNAseq). We analyzed vector biodistribution and gene transfer in PBMCs at 1-, 3-, 7-, 14- and 23-days post AAV administration, in BAL, in axillary lymph node and colonic biopsies at 7-days post AAV, and in a panel of 8 lymphoid and 10 non-lymphoid tissues at 23-days post AAV. We also determined the levels of neutralizing antibodies (NAbs) against each capsid within our AAV-inoculum at the time of injection to evaluate the degree to which pre-existing humoral immunity can influence vector biodistribution and gene transfer. Our findings show that there is significant animal-to-animal variation in both the biodistribution and tissue transduction of different AAV vector capsids, and that pre-existing serotype-specific neutralizing antibodies can play a significant role in the outcomes for each capsid once administered systemically. They also allowed us to identify capsids that were enriched in several different tissues of interest for the development of gene therapeutics including liver, heart, and muscle. In summary, this method offers a robust approach to vector optimization that can be used to identify and validate vectors for gene delivery to potentially any anatomical site or cell type.

384 E-Selectin/AAV Gene Therapy Promotes Myogenesis and Skeletal Muscle Recovery in a Mouse Hindlimb Ischemia Model

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Introduction: Chronic limb hypoperfusion due to systemic atherosclerosis in peripheral artery disease (PAD) can lead to life-limiting claudication and limb-threatening ischemia. Identifying novel targets regulating neovascularization and regeneration of ischemic tissues is key to developing nonsurgical treatment adjuncts for PAD. E-selectin is an adhesion molecule that mediates recruitment of endothelial progenitor cells during neovascularization. Therapeutic priming of ischemic limb tissues with intramuscular E-selectin gene therapy promotes angiogenesis and reduces tissue loss in a mouse hindlimb gangrene model. In this study, we further evaluated the effects of E-selectin gene therapy on skeletal muscle recovery, specifically focusing on exercise performance and myofiber regeneration. **Methods:** C57BL/6J mice aged 10-12 weeks were treated with a total of $1x10^{11}$ viral genome of either E-selectin/adeno-associated virus serotype 2/2 gene therapy (E-sel/AAV) or LacZ/AAV as control. Hindlimb ischemia was induced by unilateral femoral artery and vein coagulation. Recovery of hindlimb perfusion was measured by laser Doppler perfusion imaging and skeletal muscle function assessed by treadmill exhaustion and grip strength testing. On postoperative day (POD) 21, gastrocnemius muscles were harvested for immunofluorescence analysis of myogenic precursors and fiber-type distribution. Expression of *E-sel* transgene was confirmed by real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). **Results:** After induction of hindlimb ischemia, both E-sel/AAV- and LacZ/AAV-treated mice experienced a similar reduction in perfusion (0.08 ± 0.01 *vs* 0.08 ± 0.01, $P = .64$). Hindlimb perfusion progressively recovered in both groups but was significantly enhanced by E-sel/AAV gene therapy as early as POD 3 (0.21 ± 0.02 *vs* 0.14 ± 0.01, $P = .002$) and at all time points through POD 21 (0.58 ± 0.02 *vs* 0.33 ± 0.02, $P < .001$). In parallel with recovery of hindlimb perfusion, mean grip strength was significantly greater in mice treated with E-sel/AAV compared to LacZ/AAV starting on POD 7 (1.89 ± 0.08 *vs* 1.57 ± 0.07 gf/g, $P = .009$) and through POD 21 (2.36 ± 0.08 *vs* 1.93 ± 0.09 gf/g, $P = .001$). E-sel/AAV-treated mice also outperformed LacZ/AAV controls during treadmill exhaustion testing on POD 7 (264 ± 26 *vs* 157 ± 23 m, $P = .009$) and through POD 21 (354 ± 27 *vs* 232 + 30 m, $P = .009$). On POD 21, RT-qPCR confirmed that *E-sel* mRNA levels were 322-fold higher in muscle treated with E-sel/AAV compared to LacZ/AAV. Immunofluorescence analysis demonstrated enhanced proliferation of myogenic precursors as measured by number of $MyoD^+$ cells (10.3

± 2.1 *vs* 1.5 ± 0.4 cells/mm^2, P = .003), Ki-67$^+$ cells (5.7 ± 0.9 *vs* 1.7 ± 0.2 cells/mm^2, P = .002), and MyoD$^+$/Ki-67$^+$ co-staining cells (1.8 ± 0.7 *vs* 0.03 ± 0.03 cells/mm^2, P = .040), as well as a greater proportion of Myh7$^+$ myofibers (21.0 ± 0.7% *vs* 4.9 ± 1.5%, P <.001) in ischemic muscle treated with E-sel/AAV compared to LacZ/AAV. **Conclusion:** First, this study confirms the efficacy of intramuscular E-sel/AAV gene therapy for therapeutic angiogenesis in a mouse hindlimb ischemia model. In addition to improving perfusion, E-sel/AAV also enhances proliferation of myogenic precursors and is associated with increased proportion of type I/slow-twitch myofibers in regenerating ischemic muscle. Altogether, these effects correlate with improved exercise capacity and suggest a potential role for E-sel/AAV gene therapy as a nonsurgical option in patients with life-limiting PAD.

385 *In Silico* Prediction and *In Vivo* Testing of Promoters Targeting GABAergic Inhibitory Neurons

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Impairment of GABAergic inhibitory neuronal function is linked to epilepsy and other neurological and psychiatric disorders. One therapeutic strategy for treating disorders where GABAergic inhibition is impeded, is to enhance GABA neuronal activity in the brain. Recombinant adeno-associated virus (rAAV)-based gene therapy targeting GABAergic neurons is a promising treatment for GABA-associated disorders. However, there is a need to develop rAAV-compatible gene regulatory elements capable of selectively driving expression in GABAergic neurons throughout the brain. Here, we designed several novel GABAergic gene promoters. *In silico* analyses, including evolutionarily conserved DNA sequence alignments and transcription factor binding site searches among GABAergic neuronal genes, were carried out to reveal novel sequences for use as rAAV-compatible promoters. rAAVs (serotype 9) were injected into the CSF of neonatal mice, and into brain parenchyma of adult mice to assess promoter specificity. In mice injected neonatally, transgene expression was detected in multiple brain regions with very high neuronal specificity, and moderate to high GABAergic neuronal selectivity. The GABA promoters differed greatly in their levels of expression, and in some brain regions, showed strikingly different patterns of GABAergic neuron transduction. This study is the first report of rAAV vectors that are functional in multiple brain regions using promoters designed by *in silico* analyses from multiple GABAergic genes. These novel GABA targeting vectors may be useful tools to advance gene therapy for GABA-associated disorders.

386 Preclinical Studies with HMI-104, an AAVHSC Vectorized C5 Monoclonal Antibody, for the Treatment of PNH

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Adeno-associated virus (AAV) gene therapy has the potential to offer a long-term resolution for diseases that rely on chronic dosing of therapies such as monoclonal antibody-based drugs. Our Gene Therapy-mAb (GTx-mAb) platform is focused on using the liver to express a C5 mAb with a one-time dose. HMI-104 is an AAVHSC vector designed to elicit hepatic expression of a C5 monoclonal antibody (C5mAb) for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and other complement-mediated disorders. HMI-104 is delivered via a single intravenous (I.V.) injection and the expression of C5mAb can inhibit C5-mediated lysis of red blood cells. We have previously shown that a platform GTx-mAb construct achieved dose-dependent and sustained expression of functional C5mAb in two immunocompromised models: NOD SCID mice, which lack murine C5, and FRG® liver-humanized mice, which express physiological levels of human C5 (Sharma et al, ASGCT-2021). Here, we present the results from two nonclinical studies with HMI-104 for the treatment of PNH. The 16-week dose range-finding study of HMI-104 in NOD SCID male mice was designed to determine the relationship between dose levels, liver vector genomes (vgs), mRNA levels, C5mAb serum concentrations, and *ex vivo* hemolysis over time. In the study, HMI-104 led to sustained antibody levels with serum C5mAb concentrations steadily increasing through Week 5 and plateauing thereafter to Week 16 (end of study). A dose-dependent increase in liver vg concentrations and liver transcript levels were shown for all doses and time points. The 4-week, dose range-finding study in the humanized liver FRG KO mouse xenograft model was designed to evaluate HMI-104 transduction (vgs) and mRNA levels in human hepatocytes, and to assess C5mAb levels in the presence of human C5. In the study, HMI-104 transduced human hepatocytes *in vivo* and expressed sustained serum C5mAb levels in the presence of human C5, achieving antibody levels by Week 3 that were comparable to those obtained at steady state in the NOD SCID mouse model. Finally, we established that C5mAb following HMI-104 administration in the humanized liver FRG and NOD SCID models is functional, as determined by complete inhibition of *ex vivo* hemolysis. In conclusion, we present nonclinical data with HMI-104 demonstrating sustained expression of functional C5mAb levels in NOD SCID and humanized liver FRG KO mice. These results support the development of HMI-104, which is currently in IND-enabling studies, for the treatment of PNH and complement-mediated disorders. Given the severity of PNH and the unmet need associated with available therapies, HMI-104 aims to provide, with a single treatment, sustained serum C5mAb sufficient to inhibit C5 complement-mediated lysis and reduce breakthrough and residual intravascular hemolysis associated with insufficient C5 antibody levels in PNH patients.

387 Anticodon-Engineered Transfer RNA for the Treatment of Nonsense-Associated Cystic Fibrosis

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Cystic fibrosis (CF) is an autosomal recessive disorder caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene encoding CFTR anion channel. CFTR dysfunction disrupts chloride and bicarbonate transport across epithelia and results in the buildup of thick and sticky mucus in multiple organs. Among different CF-causing mutations, nonsense mutations, which result from single nucleotide conversion of a sense codon into a nonsense or premature termination codon (PTC), account for ~10% of CF cases. PTCs lead to significant reduction in steady-state transcript expression through nonsense-mediated mRNA decay (NMD) process and production of a truncated, nonfunctional protein. Because CF patients with nonsense mutation have a problem with protein synthesis, they are not responsive to the FDA-approved CFTR modulator drugs that target the malfunctioning CFTR protein. Anticodon-engineered transfer RNA (ACE-tRNA) is a gene therapy approach being investigated in our lab for this patient population. We previously reported that liposome-based delivery of ACE-tRNA encoding cDNAs efficiently suppresses PTCs, inhibits NMD and rescues >70% of functional CFTR channel encoded from its endogenous gene. In this study, we tested the efficiency of adenovirus (Ad)-delivered ACE-tRNAs to rescue endogenous CFTR mRNA and protein expression and determined the benefit of ACE-tRNA delivery in combination with NMD inhibitor and the FDA-approved CFTR modulators. Further, we wanted to determine the ability of one ACE-tRNA sequence to rescue multiple nonsense-associated CF mutations. ACE-tRNAs delivered to 16HBEge cell lines with common CF-causing nonsense mutations significantly increased CFTR transcript level, which was measured by quantitative reverse transcription PCR. Significant ACE-tRNA-dependent rescue of functional CFTR channels was also measured by Ussing chamber recordings. NMD inhibitor treatment improved CFTR transcript expression, but rescue of CFTR channel function was only demonstrated when combined with ACE-tRNAs. In contrast, treatment with CFTR modulators had no effect on CFTR mRNA level but had synergistic effects on CFTR channel activity with ACE-tRNAs. Excitingly, we discovered the possibility of advancing one ACE-tRNA sequence as a platform therapy for several nonsense CF variants. In summary, ACE-tRNAs, especially in combination with other small molecule drugs targeting the overall transcript level and/or protein stability and function, hold great promise as a novel therapeutic option for CF patients with nonsense mutations.

388 Sensor-Actuator-Modulator Gene Therapy for Glaucoma

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PURPOSE Glaucoma is characterized by overexpression of fibrous proteins in glaucomatous trabecular meshwork (TM) leading to increased stiffness of TM and associated poor drainage of Aqueous humor (AH) through the conventional outflow pathway. Glaucoma is recalcitrant to treatment and the resulting optic nerve damage is irreversible. The currently available pharmacological and surgical treatments for glaucoma have significant limitations and side-effects, which include, systemic reactions to medications, patient non-compliance, eye infections, surgical device failure, and damage to the eye. Here, we put forth a novel commission for sensor-actuator-modulator (SAM) channel as a virally delivered transgenic pressure modulator in the impaired TM of glaucomatous eyes. **METHODS** Pressure-clamp electrophysiology was conducted to evaluate SAM functioning in cells in-vitro. Different glaucoma mice models were used to evaluate the therapeutic efficacy of SAM gene therapy. AAV-SAM was delivered to TM via intracameral injection. Intraocular pressure (IOP) and outflow measurements were carried out at baseline and different post-injection time points. Structural changes were monitored via optical coherence tomography (OCT), and QPCR was used for biodistribution. The safety was analyzed via ELISA and immunohistochemistry. **RESULTS** Our results demonstrate that SAM is: (i) functional in cultured TM cells, (ii) successfully transduced *in vivo* in TM cells; and (iii) effective in lowering the IOP in a mouse model of ocular hypertension without (i) non-targeted expression (measured by QPCR); (ii) ocular damage (assessed by OCT); (iii) inflammatory cytokines in plasma (detected by ELISA); or (iv) immune cell response and (v) loss of viability of targeted cells expressing SAM over long period. **CONCLUSION** The use of heterologously expressed engineered SAM channel, acting as macromolecular pressure sensor and outflow actuator in TM cells, led to regulation of IOP. The development of a safe effective long-lasting single dose therapeutic for the treatment of Primary Open Angle Glaucoma would be transformative in ophthalmology and would improve the lives of millions of people worldwide.

389 Extracellular Vesicles for Delivery of AAV Vectors

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Introduction: Adeno-associated viral (AAV) vectors are a leading platform for gene therapy however, the biggest hindrance to their clinical application is the host immune response that generates neutralizing antibodies (NAb) following AAV administration. The presence of NAb prevents re-administration of AAVs, but also their application in seropositive patients. The estimate is that 40 - 80 % of the adult population is seropositive due to natural exposure to the wild-type virus. During standard AAV vector production, a portion of AAV particles released into the cell culture media is associated with extracellular vesicles (EVs) - membrane-bound vesicles released into the extracellular environment by cells. We hypothesize that these EV-associated AAVs (EV-AAVs or exoAAVs) will provide complete or partial protection from NAbs, allowing for repeated administration and treatment of seropositive patients. However, due to the similar physical properties of EVs and AAVs (i.e size and density) separating exoAAVs from free AAV particles present in the cell culture media is

quite challenging. Therefore, we aimed to assess different protocols for the isolation/purification of exoAAVs to determine which one is the most effective in removing the free AAV particles from final exoAAV samples. Moreover, exoAAVs obtained using the most and the least successful purification protocol were tested in vivo to assess their transduction efficiency and immunogenicity. ***Methods:*** Standard AAVs and exoAAV vectors were produced by triple transfection of HEK293T cells and isolated 72h post-transfection from harvested cell lysates and culture media, respectively. Standard AAVs were isolated using iodixanol gradient centrifugation. For isolation of exoAAVs five different protocols were tested: differential ultracentrifugation (UC), size exclusion chromatography (SEC), differential gradient centrifugation (DGC), and two combined protocols using two isolation methods, combined protocol 1 and 2 (CP1 and CP2). 7 weeks old male CD-1 mice were injected IV with standard AAV, UC exoAAV, and CP2 exoAAV samples, dosed at $3*10^{11}$ genome copies per animal. 2 weeks post-injection animals were sacrificed and different organs and plasma samples were collected for assessment of transduction efficiency and presence of NAb, respectively. ***Results:*** The presence of EVs and AAV particles was confirmed in all exoAAV samples using different characterization methods. AAV capsids enveloped by lipid membranes were observed in all exoAAV samples using cryoTEM however, the contamination of samples with free AAV particles was the highest in samples purified using UC (most often used for isolation of exoAAVs) while it was the lowest in the samples isolated using combined protocols. Tested in vivo, exoAAVs purified using CP2 showed lower transduction efficiency compared to standard AAVs and exoAAVs purified using UC. On the other hand, cell-based NAb assay indicated lower levels of NAb in the plasma of animals injected with exoAAV sample isolated by CP2. ***Conclusions:*** The combined protocols outperform the UC protocol in separating exoAAVs from free AAV particles present in starting cell culture media. Tested in vivo, exoAAVs isolated by CP2 display lower transduction efficiency but also lower immunogenicity compared to standard AAV and UC isolated exoAAVs.

390 Purification of a Broad Range of Adeno Associated Virus Serotypes from HEK293 Cell Lysates via the Peptide Based Affinity Adsorbents

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Cell and gene therapies (CGTs) are at the forefront of innovation to treat rare degenerative & dysregulation diseases, immunological disorders, and genetic defects diseases, such as A1AT deficiency. To support the manufacturing of Cell and Gene Therapies, we have developed innovative peptide-based affinity ligands, specifically designed to capture a wide-ranging Adeno-Associated Virus serotypes (AAVs) from HEK293 cell lysate with excellent yield and binding capacity. This work presents the development of short synthetic AAV-binding peptides and demonstrated their binding reactivity towards a set of AAV serotypes that includes AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, AAV9, and AAVrh10, as well as synthetic serotypes. Candidate AAV-targeting peptides were identified by screening a solid-phase peptide library against a mixture of AAVs spiked in HEK293 lysate via microfluidic screening technology. Twelve promising AAV-binding ligands were investigated, some of which showed high binding capacity toward AAVs with good salt-tolerance ($>1x10^{13}$ genome copies/mL of resin), mild elution (pH 6.0, 0.4 M $MgCl_2$) with excellent recovery (>38%), comparable purity ($LRV_{HCPs} > 1$), good chem-stability (over 20 runs) and low immunogenicity caused by ligand leakage. Notably, the AAVW2 ligand can currently purify the AAV2, AAV6, AAV8 and AAV9 serotypes from TFF-treated HEK293 cell lysate with excellent yield of 63.2%, 57.7%, 58.1%, and 38.2%, respectively. In sum, affinity peptide ligands enable a robust, fast, and platformable purification process for a broad range of AAV serotypes with excellent yield in one chromatography step.

391 Downstream Purification: A Toolbox Approach for Adaptive Multi-Serotype AAV Solutions

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There are several key attributes to consider when developing a process for purification of AAV viral vectors. At Pharmaron, we have successfully developed a downstream platform process for the purification of such vectors but with an adaptive toolbox to manage these varying attributes. Through careful selection of the best techniques, we can handle a wide range of serotypes, genes of interest, harvest yields, impurity profiles and titres of different gene therapy products. We utilise our high throughput DSP robotics platform to conduct an early assessment of a products fit to our platform process with speed and accuracy. Once assessed, we can focus on a targeted optimisation approach to determine, the best resin, elution conditions, chromatography approach or filter type to maximise process yield. We continue to develop our process through evaluation of new technologies, offering significant improvement on standard 'one platform fits all' approaches. Our flexibility to employ different tools from the toolbox, allows us to develop advanced technical solutions to a wide range of gene therapy product needs.

392 Advancing AAV: Novel Sequencing Solutions for Quality Control in Gene Therapy

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Interest in cell and gene therapy-based disease prevention and treatment has grown rapidly over the last decade, however there are still hurdles to overcome and progress to be made in the field. Recombinant adeno-associated viruses (rAAV) have become the vector of choice for virus-mediated gene therapy due to their non-replicating nature, high-titers, low immunogenicity, and low genotoxicity. Extensive quality control (QC) throughout the entire development and manufacturing process is essential. A robust QC process expedites safe and effective commercialization of the final product. Next-generation sequencing (NGS) offers an effective high-throughput approach for monitoring AAV quality, from initial construct assembly to analysis of the encapsulated product. Both Illumina® short-read and PacBio® long-read

sequencing technologies offer distinct advantages including sequencing of the entire AVV genome and inverted terminal repeat (ITR) regions, with detection of potential mutations, truncations, and contaminants. Both platforms require conversion of the single-stranded genome to double-stranded DNA prior to library preparation; however, high-fidelity protocols for this step are lacking. Additionally, an efficient bioinformatics pipeline is needed to generate interpretable results from massive amounts of NGS data. Here we describe our novel proprietary workflows, starting with sequence confirmation and correction of AAV plasmid, through QC results using both NGS platforms with supported from a regulatory-compliant Sanger assay. Sanger ITR sequencing and sequence correction upstream alleviates potential downstream issues in viral packaging. The combined NGS approach alleviates current constraints for high throughput AAV sequencing and thereby enhance the overall QC process. Our Good Laboratory Practices (GLP) Sanger sequencing method extends read lengths through the entire ITR regions, allowing for rapid sequence confirmation of the final AAV product. The combination of these approaches enables a comprehensive solution, ideal for sequence confirmation of both transfer plasmid and final packaged product for improved AAV manufacturing in advance of FDA IND and BLA filings.

393 Bioanalytical Evaluation of Single-Stranded DNA for Gene Therapy Applications: A Reference Standard for Quality Control Workflow

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For research use only. Not for use in diagnostic procedures. **Background:** Single-stranded DNA (ssDNA) is a DNA donor template in adeno-associated virus (AAV)-based gene therapy. For CRISPR Homology-Directed Repair (HDR) mediated gene knock-in, ssDNA has lower rates of random or off-target integration and lower toxicity compared to double-stranded DNA. In addition, ssDNA-based aptamers with high specificity and affinity for their targets provide a new tool for drug delivery and targeted cancer therapy. Rapid growth in the use of ssDNA in biotherapeutics underscores the importance of quality control to ensure the integrity and purity of ssDNA. **Method:** RNA Pico reagent (Part#: CLS960012), and DNA 5K/RNA/CZE HT LabChip (Part#:760435) were used in the study. A novel ssDNA ladder (Part #: CLS157950, from PerkinElmer) ranging from over 1000 to over 7000 bases was evaluated on PerkinElmer's LabChip® GXII Touch™ (Part# CLS138160). Flow control in microfluidics was further optimally configured for better size resolution. **Result and Conclusion:** This report provides a reliable, higher throughput reference standard, and bioanalytical screening solution for ssDNA samples (workflow in **Figure 1**). The novel ssDNA ladder is suitable for analyzing 1.1kb to 7.2kb ssDNA samples **(Figure 2).** In addition, double-stranded DNA (dsDNA) can be efficiently detected by the new assay, with different migration speed compared to ssDNA. **Discussion:** The availability of ssDNA ladder as a reference standard is of significant value for the identification, evaluation, and characterization of ssDNA-based payloads either via viral (e.g., recombinant adeno-associated virus, rAAV) or non-viral vehicles (e.g., Lipid nanoparticles, LNPs) in gene therapy or gene editing applications. Hence, the novel size standard we report here, will be beneficial for molecular bioanalytical and quality control workflows across gene therapy and other ssDNA-related applications. For instance, it can be used effectively to evaluate critical quality attributes (CQAs) such as relative titer quantification, purity, and integrity of ssDNA from rAAV preparations (~4.7kb length).

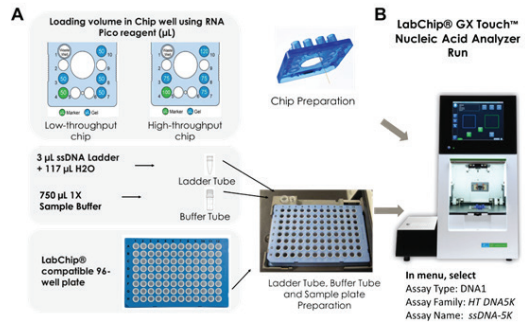

Figure 1: A workflow of ssDNA analysis assay. (A) Chip, Ladder and sample preparation. (B) Program selection on instrument.

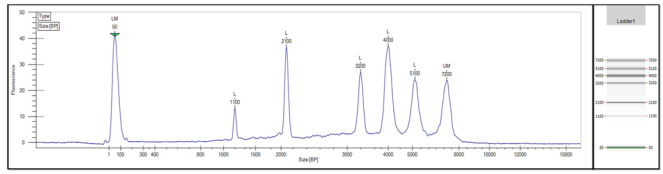

Figure 2. ssDNA Ladder Performance. Ladder working concentration used on LabChip® GXII Touch™ here is 2ng/µL diluted by nuclease-free water.

394 rAAV Produced by Sf9-Baculovirus System Shows Different Product Quality Profile Throughout the Production Cycle

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The Sf9/baculovirus expression system for rAAV production offers advantages such as higher yields and lower costs of raw materials, compared to other production systems. Generally two baculovirus expression vectors - containing either the Rep/Cap elements or the gene of interest - are utilized to co-infect Sf9 cells. The infection may occur at Low or High Multiplicity of Infection (MOI) and each process has its advantages and challenges. High MOI infection results in a high percentage of cells simultaneously co-infected so that cell division stops very rapidly. This synchronous infection could generate rAAV with more homogeneous product quality; however, high MOI infection can be costly due to large quantity of baculovirus needed. In low MOI infection a much lower quantity of virus is added to the culture and cells continue to divide until the baculovirus completely infects the culture, so that co-infection happens during secondary or consequent infection cycles.. However low MOI infection yields rAAVs that are produced over a longer period in the culture, raising the question of whether the quality of the rAAV produced is homogenous throughout the culture. In this study we evaluated the quality of the rAAV produced throughout the culture process in 12 hour intervals. ddPCR is performed to analyze

the rAAV productivity. The quality is assessed by examining VP ratios, percentage of full particles and integrity of the vector genome. rAAV productivity and product quality showed different patterns by hour and the MOI utilized, shedding light on the optimal harvest time for rAAV production.

395 Process Development Considerations for Ultrafiltration & Diafiltration of Viral Vectors

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Compared to monoclonal antibody downstream processing, viral-vector manufacturing is less standardized, which can result in lower yields and throughputs in unoptimized processes. A critical part of the downstream purification process addressed within this work is the ultrafiltration/diafiltration (UF/DF) steps which can be optimized through the proper selection of molecular weight cutoff and operating mode. Properly designed tangential flow filtration (TFF) operations can provide concentration, buffer exchange, and impurity reduction through UF/DF in the downstream manufacturing of viral vectors. Viral vectors can be retained while also removing impurities like endonucleases using a 100 or 300 kDa membrane cut-off. The preferred membrane cut-offs fall between the tighter UF (30 kDa) and high permeability microfiltration applications (>1000 kDa). TFF with tighter membrane cut-offs operate by transmembrane pressure (TMP) control where the flux and TMP are controlled by adjusting the retentate-side pressure. High permeability microfiltration applications use permeate control and rely on a pump or a flow-controlled valve on the permeate side to control the flux. This study evaluated both control modes using 100 and 300 kDa membranes by evaluating performance for a 10X concentration and 5 diavolume diafiltration of a clarified adeno-associated virus (AAV2) stream. TMP control had a flux (or processing time) advantage over permeate control, while permeate control had an impurity clearance advantage. Both control modes had similar yields (>85% recovery) of AAV2. Initial process development and optimization of parameters were performed with laboratory-scale 50 cm^2 cassette using 50-200 ml volume. Lab-scale parameters were then utilized to scale-up to larger area devices demonstrating a successful 300-fold scale-up of the operation.

396 Partial Genome Content within rAAV Impacts Performance in a Cell Assay Dependent Manner

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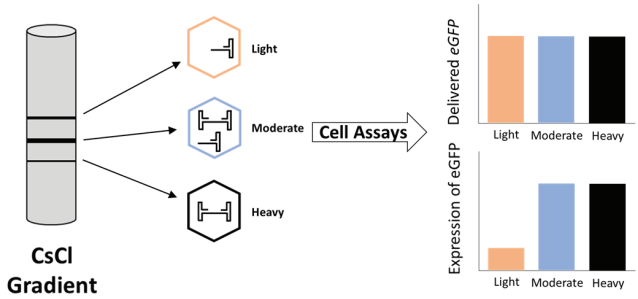
Recombinant adeno-associated viruses (rAAV) deliver DNA to numerous cell types. However, the packaging of partial genomes into the rAAV capsid is of concern. Although empty rAAV capsids are studied, there is little information regarding the impact of partial DNA content on rAAV performance in controlled studies. To address this, we characterized and tested vectors containing varying levels of partial, self-complementary *eGFP* genomes. Density gradient cesium chloride ultracentrifugation was used to isolate three distinct rAAV populations: 1) a lighter fraction, 2) a moderate fraction, and 3) a heavy fraction. Alkaline gels, SEC-MALS, and CD-MS were used to characterize the genome size of each population and ddPCR to quantify residual DNA molecules. Cell assays demonstrated opposing results depending on the endpoint measurement. Both live cell imaging and eGFP ELISA assays demonstrated reduced expression following transduction with the light fraction compared to the moderate and heavy fractions. However, PCR-based assays showed that the light density delivered DNA to cells at a similar efficiency as the moderate and heavy fractions. These data support the growing evidence that the genome purity impacts vector performance. Moreover, these results highlight the benefit of combining expression data with quantification of delivered DNA in cell assays.



397 Use of the GH329 Cell Line for Adeno-Associated Virus Production

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Human adenovirus derived vectors are a frequently used delivery vehicle for human gene therapy and vaccination. Much effort has been devoted to improving E1 complemented cells by limiting homology between E1 deleted adenoviral vectors and thus preventing the formation of replication-competent adenoviruses (RCA). In pursuit of this strategy PER.C6, SL0003 and GH329 were among the cells derived from the parental cells human embryonic retinoblast, A549 and HeLa respectively, that stably express E1 locus and avoid RCA formation. No reported attempts have been made to prove these cells can produce adeno-associated viruses. We demonstrate that GH329 cells enable the production of rAAV (recombinant AAV) by the three plasmid transfection method. FIG. 1 shows rAAV viral genomes measured in GH329 compared to parental HeLa cell (measured value below limit of detection) after triple transfection. We are investigating ways to improve the properties of GH329 cells and will present our ideas and relevant results. The resulting cell line could improve production methods and address yield, scalability, and ethical concerns in rAAV production.

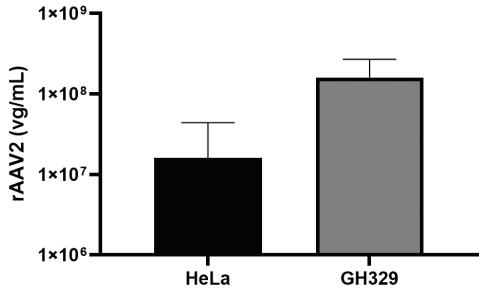

FIG. 1 Digital PCR (QIAcuity, Qiagen) measurements of rAAV2 viral genomes. GH329 $1.6x10^8$ +/- $1.10x10^8$, HeLa below level of detection.

398 Development of Chromatography Based Separation of Empty and Full Capsids in rAAV Manufacturing Process

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Recombinant adeno-associated virus (rAAV) vectors are one of the most promising technologies to deliver therapeutic transgenes for gene therapy. For optimal potency and safety, rAAV vectors must be purified from cellular and process contaminants during the downstream purification steps. During production of AAV vectors, two distinct predominant species of vector are formed, one being capsid that does not contain the therapeutic target gene, referred to as 'empty' vector, and the other being capsid that contains the therapeutic target gene, referred to as 'full' vector. Large amounts of empty or partially-filled capsids are known to cause a negative immune response in patients and reduce infectivity of full AAV. It is therefore necessary to remove the 'empty' vector from the final drug product through full capsid enrichment. Historically, gradient based empty and full capsid separation utilizing ultracentrifugation has been the industry standard, however this process is difficult to scale and perform robustly at larger scales in an end-to-end closed process that are typically performed during commercial manufacturing. A common gradient based ultracentrifugation process utilizes cesium chloride, which requires additional testing for residual cesium and toxicity levels in the final drug product. Chromatography based purification allows for a rapid and cost-efficient process for scale-up while both decreasing processing time and increasing product purity. At Forge Biologics, we have developed a robust anion exchange (AEX) chromatography method for the Full Capsid Enrichment. The AEX process can accomplish this separation by utilizing the slight difference in capsid charge between empty capsid and full capsid. Through systematic screening of 4 different AEX columns in conjunction with different elution buffer matrices, Forge has successfully developed and scaled an AEX process for several serotypes such as AAV9, AAV8, rH10, and AAV6. The rAAV purified by AEX was analyzed for vector titer by ddPCR, and for empty capsid and full capsid ratio by Analytical Ultracentrifugation (AUC) and orthogonal techniques. The data from this study will be presented, which emphasizes the need to explore the use of chromatography-based enrichment techniques in rAAV purification.

399 Single-Use Capsule versus Hollow Fibers for Ultrafiltraion/Diafiltration (UF/DF) in Viral Gene Therapy Manufacturing

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Recent advances in novel modalities, such as viral gene therapies (VGT), are driving the implementation of single-use manufacturing technologies to improve speed-to-market and manufacturing flexibility. Maximizing viral yield at each downstream purification step while also minimizing processing time is critical as VGT streams are expensive and can be sensitive to shear and exposure to ambient conditions. Tangential flow filtration (TFF) modules (e.g. hollow fibers, cassettes, and spirals) with appropriate molecular weight cutoffs (e.g. 100 and 300 kDa) for retaining viruses (e.g. AAV, Lentivirus) while removing impurities (e.g. host cell protein, DNA, and endonuclease) are an attractive purification technology if properly implemented. Single-use TFF modules address the need to reduce process complexity and increase manufacturing flexibility while minimizing product and operational risks. Herein, we compare traditional single-use TFF hollow fiber modules to a new pre-sterilized, holder-less and linearly scalable spiral module design. Using model and real AAV streams, concentration and diafiltration performance characteristics including scalability, process efficiency/time, and viral yield are compared. At the same feed flow rate, the single-use capsule has a higher and more stable operating flux with comparable virus yield compared to hollow fiber modules, resulting in smaller systems and/or faster TFF operations. As such, the capsule provides a more productive single-use TFF process than hollow fiber modules, enabling high concentration and/or diafiltration targets in less time while maintaining the same high viral yields.

400 A Streamlined Downstream Purification Platform Process for Adeno-Associated Viral Vectors

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[1]Research & Development, Lonza, Houston, TX,[2]Process Development, Lonza, Houston, TX

One major obstacle to achieving shortened development times and reduced costs of adeno-associated gene therapy products is the lack of standardized and cGMP-compliant downstream purification platforms. Besides providing high yields and purities, such platforms should be applicable to a wide variety of AAV serotypes as well as being customizable with minimal process modifications to allow rapid development of scalable downstream purification processes for clinical testing. Previously, we developed a comprehensive AAV production platform consisting of a proprietary high-producer suspension HEK293 cell line, a high-performing packaging plasmid system and an optimized triple transfection-based upstream process. In this study, we developed a streamlined downstream purification process using a GFP-encoding AAV9 construct (AAV9-GFP) as model. The applicability of the process was subsequently verified for several

other AAV serotype/GOI combinations. For AAV9-GFP, the total process recovery was up to 38 %, starting from unclarified cell lysate at 3 L bioreactor scale, with post lysis titers of ~1.0 E+15 vg per liter cell culture by ddPCR titer assay. After affinity capture, circa 60 % full capsid content were obtained, which could be further enriched to up to 74-90% full, based on AUC capsid only quantification and/or SEC-MALS or mass photometric analyses. For an AAV9 model construct encoding a therapeutically relevant protein (AAV9-GOI1), a post-lysis intact genomic titer by 2D ddPCR of 4.1E+14 vg per liter production scale was achieved. After affinity capture, a Full:Empty ratio of 1.1 was obtained which increased to 11.5 for the BDS, with the final product containing 86 % full and less than 8 % empty capsid (AUC capsid only quantification). Starting from crude cell lysate, an overall downstream process yield of 33 % was obtained, while residual host cell DNA was reduced to below 2.5 ng/E+12 vg and HCP levels below the assay's lower limit of quantification. In conclusion, we developed a streamlined AAV downstream purification process with high total recovery and full% capsid content of the final product at high levels of purity.

401 Sensitivity of AAV to Chemical and Physical Changes from Different Degradation Pathways

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Gene therapy has been successfully applied in the clinic using adeno-associated virus (AAV) vectors to treat genetic diseases. AAV vectors are exposed to a variety of conditions which may cause product degradation over the product lifecycle, but the sensitivity of vectors to these different conditions remains poorly understood. Improved understanding of these degradation modes will allow us to design better manufacturing processes and formulations to maximize the safety and efficacy of AAV vectors. Formulated AAV2 drug product was subjected to various physical, thermal and chemical stresses for multiple durations of time to generate a library of degraded samples. Samples were characterized and product quality attributes relevant to stability were measured. The impact of each stress on the product quality was assessed.

Degradation Conditions and Durations		
Stress	**Condition**	**Duration**
Physical	-80°C/Room Temperature (RT), Freeze-thaw, small volume	Up to 10x cycles
	-80°C/RT, Freeze-thaw, large volume	Up to 3x cycles
	Photooxidation, fluorescent laboratory light at RT	Up to 7 days
Thermal	40°C	Up to 7 days
Chemical	Acidic, pH 2.0 at RT	Up to 7 days
	Acidic, pH 4.0 at RT	Up to 7 days
	Alkaline, pH 8.0 at RT	Up to 7 days
	Alkaline, pH 10.0 at RT	Up to 7 days
	Chemical oxidation, 1 ppm H_2O_2 at RT	Up to 24 hours
	Chemical oxidation, 3% H_2O_2 at RT	Up to 24 hours

Changes in product quality were detected from nearly all evaluated conditions. Some conditions caused only physical changes to the product (particle size distribution or precipitation) while other conditions caused additional chemical changes (both protein and genome). Decreases in potency were observed along with physical and chemical changes to the product. AAV2 was most sensitive to extreme acidic conditions and high concentrations of chemical oxidants. High temperature also caused physical and chemical changes. AAV2 was less sensitive to alkaline conditions, although very high pH caused notable product degradation. Freeze-thaw cycling caused physical changes to the product, but these changes were much more notable with larger fill volumes. AAV2 was not highly sensitive to photooxidation or low concentrations of chemical oxidants. Notably, the capillary electrophoresis sodium dodecyl sulfate (CE-SDS) method used to quantify vector proteins was not capable of detecting any changes to the product from any of the degradation modes evaluated and the effectiveness of size exclusion chromatography (SEC) to measure aggregation was limited. These results identify the extremes and limits which AAV2 can be exposed during manufacturing and product handling and may also help diagnose future product stability issues. This work further established whether analytical methods used to measure quality attributes are stability indicating.

402 Fit-for-Purpose Assay Characterization of GS-100 Potency Assay

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[1]Grace Science, LLC, Menlo Park, CA,[2]Integrated Analytical Solutions, Inc, Berkeley, CA

Introduction: N-glycanase 1 (NGLY1) Deficiency is an ultra-rare autosomal recessive disorder caused by the loss of NGLY1 function. GSL is developing GS-100, an AAV9 gene replacement therapy designed to deliver a fully functional copy of the human NGLY1 gene, for the treatment of NGLY1 Deficiency. NGLY1, a cytosolic enzyme, catalyzes the hydrolysis of the amide bond between N-linked glycans and proteins. When NGLY1 is not sufficiently functional its substrate GlcNAc-Asn (GNA) accumulates. Thus, GNA serves as a critical biomarker of pharmacodynamic response to GS-100. The GS-100 potency assay precision, accuracy, linearity, range, and specificity were assessed and described here. Aggregation and potency were assessed to determine if higher concentrations of GS-100 were associated with decreased potency. Methods: *GNA Quantification Methods* NGLY1 Knock Out (KO) cells overexpressing the AAV receptor were transduced in low serum conditions at various multiplicities of infection (MOI), incubated, harvested, frozen, homogenized, extracted, and analyzed by hydrophilic interaction chromatography (HILIC) and MS/MS against a standard curve. Detection and accuracy controls were assessed in a wild type control (low GNA) or untransduced KO control (high GNA). The Activity is reported as % reduction (%R) of GNA relative to untreated NGLY1 KO cells per log (MOI). The Mean Activity is determined by averaging the %R/log(MOI) across two or more MOIs per experiment. Accuracy was confirmed by WB and qRT-PCR. *Sample Concentration and Aggregation Testing:* Manufacturing batch samples were titered by ddPCR targeting NGLY1. Samples were concentrated using

ultra- or centricon centrifugation and then re-titered to confirm the concentration. Samples were analyzed via Stunner-DLS or HPLC-SEC. Results: The GS-100 assay passed all performance parameter criteria.

Performance Parameters	Acceptance Criteria	Result
Inter-Assay Precision	Activity ≤ 30% CV for each MOI Mean Activity ≤ 30% CV	0.23% to 18.5% 8.21%
Intra-Assay Precision	Activity ≤ 30% CV for each MOI	1.79% to 12.76%
Accuracy to mRNA expression	Correlation between mRNA fold change and Activity should have an $r^2 > 0.9$	$r^2 = 0.9510$
Accuracy to Western blot analysis of protein expression	Dose dependent response of NGLY1 protein band in Western blot analysis	0 >10k > 100k > 1MM
Linearity of LC-MS/MS	$r^2 > 0.9$ across GNA range spiked into WT background (GNA ng/mL)	$r^2 = 0.9932$
	GNA ng/mL ≤ 30% CV at each concentration	1.2% to 9.2%
Linearity of KO/WT Cell Mixing	$r^2 > 0.9$ across 0-100% KO cells	$r^2 = 0.9886$ and 0.9632
Range	Transduced *NGLY1*$^{-/-}$ cells should have GNA concentration values that do not exceed the KO cells, but reach or exceed the GNA concentration in WT cells	KO Untreated: 148.33 ng/mL KO Treated Cells: 24.30 - 104.17 ng/mL WT Untreated: 50.23 ng/mL
	Activity ≤ 30% CV at each MOI	2.61% to 23.86%
Specificity	AAV9-GFP should not significantly decrease GNA ng/mL	AAV9-GFP did not statistically decrease GNA ng/mL

Aggregation and Potency Assessments

Virus	DLS (nM)	SEC HPLC		GOI Titer (vg/mL)	Activity %R/log(MOI)
		% Aggregate	% Monomer		
Vendor 1 – Lot 1	29.25	0	100	2.00E14	15.14
Vendor 1 – Lot 2	33.96	0	100	1.87E14	13.34
Vendor 2 – Lot 1	31.57	3.2	96.8	9.70E12	12.17
Vendor 2 – Lot 1, concentrated	27.41	NT	NT	5.37E13	13.82
Vendor 2 – Lot 1, concentrated	32.37	11.35	88.65	9.22E14	14.62

NT = Not tested

Conclusions: The GS-100 potency assay showed acceptable assay performance and was deemed suitable for use in fit-for-purpose analysis of GS-100 potency comparability across manufacturing batches. GS-100 was concentrated from 9.70E12 - 9.22E14 vg/mL and potency and aggregation was determined for each lot. There was no loss in potency, suggesting that GS-100, an AAV9-hNGLY1 gene therapy, is potent at a wide range of concentrations.

403 A Leading Engineered Capsid Harboring a 7-mer Peptide Insertion Yields Low Titers and Packages Heterogeneous Vector Genomes

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[1]UMass Chan Medical School, Worcester, MA,[2]Co-correspoding Authors, Worcester, MA

Recombinant adeno-associated virus (rAAV) vectors have become the ideal human gene therapy vehicle. However, there are still some barriers hindering their expanded application. For example, rAAV administration via intraocular injection can trigger vasculitis or uveitis in response to foreign agents. These adverse events can typically be suppressed by anti-inflammatory drugs, but they remain leading factors in negatively impacting safety and therapeutic efficacies. AAV2.7m8 is an engineered capsid that carries a unique seven amino acid insertion at each of the three-fold spikes of AAV serotype 2 (AAV2). AAV2.7m8 is considered a leading capsid for transducing photoreceptor cells; however, we have consistently observed that it packages with low titers irrespective of the transgene cassette used. To illustrate this phenomenon, we packaged via plasmid transfections of HEK293 cells, multiple transgene cassettes in self-complementary (scAAV) or single-stranded AAV (ssAAV) vectors with AAV2.7m8 or AAV2 capsids. We found that during the purification of vectors by CsCl density gradient ultracentrifugation, preparations using AAV2.7m8 capsids consistently yielded undefined bands. This finding indicated that AAV2.7m8 preparations package a high degree of non-unit length genomes. To validate this interpretation, we subjected purified vectors to AAV-GPseq analyses. We found that AAV2.7m8-packaged vector genomes were consistently more heterogeneous than AAV2-packaged genomes, regardless of the vector transgene cassette used. Multi-dimensional Droplet Digital PCR analysis also confirmed these trends. In order to understand the impact of delivering heterogeneous vectors, we administered AAV2 or AAV2.7m8 vectors into mouse eyes by intravitreal injection. We found that AAV2.7m8-mediated transduction substantially stimulates infiltration of microglia, while the response to AAV2 was much milder. Further investigation into the mechanisms underpinning the delivery of heterogenous vector genomes into retinal tissues and its resulting contribution towards retinal inflammation is ongoing. Our work uncovers for the first time that differences in AAV capsid designs can directly impact vector genome heterogeneity, which in turn, can possibly trigger immune responses. We are currently exploring whether the specific group of capsids classified by 7-mer peptide insertions have a greater penchant for compromised vector genome packaging, and whether they are more immunogenic following intravitreal administration.

404 Developing a Scalable Full Empty Separation Step for an AAV Capsid by Anion Exchange Chromatography in Step Gradient Elution Mode

Regina Staffler[1], Eleni Tsiaousi[1], Johanna Weizenegger[1], Jared Babic[1], Bilal Alkharrat[2], Christina Weiss[1], Matthias Boscher[1], Johanna Wagner[1], Sinisa Petrik[1], Sebastian Ritter[1], Christine Zach[1], Marie Lott[1], Tobias Kloetzer[1], Anita Heinlein[1], Matthias Gora[1], Ahmed Youssef[1], Alexandra Schoberth[1], Melanie Langhauser[1]

[1]Ascend GCTx, Martinsried, Germany,[2]Roche, Planegg, Germany

Packaging heterogeneity in recombinant adeno-associated virus (rAAV) manufacturing may lead to potential product-related impurities like 'empty' capsids, capsids containing partial vector genomes, and capsids containing different DNA impurities. It is technically very challenging to reduce or eliminate 'empty' capsids in down-stream processing, especially in a scalable manner. Here we describe a fully scalable method to efficiently enrich the percentage of full capsids by anion exchange chromatography. An anion exchange (AEX) chromatography step was established in isocratic elution mode and optimized by design of experiment (DoE) studies. After an initial screening of different resins, a strong anion exchanger was selected for further development in step gradient mode.

DoE data were evaluated using chromatography UV and Stunner® (Unchained Labs) data. The DoE center point run results were confirmed by qPCR (recovery data) and mass photometry analysis (enrichment and percent full data). Based on the DoE studies it was determined that the capsid load and salt concentration during the empty particle elution phase are the most critical process parameters to achieve a high percentage of full particles and a satisfactory yield. The scalability of the AEX step was demonstrated by polishing material from an entire 50-liter scale run applying the optimized step gradient conditions. The enrichment factor was dependent on the proportion of 'full' and 'empty' capsids in the load material. We obtained an up to 3-fold enrichment of full capsids compared to input material, or about 75% 'full' determined by mass photometry at a vector genome step recovery of ~70%. The AEX chromatography process is designed to be optionally plugged into downstream processing depending on required product specifications. It is readily scalable and represents a tool that can be adapted for specific expression cassettes or capsids.

405 Design of Experiment (DoE) Based High Throughput Process Optimization for rAAV Manufacturing Development

Rupert Derler, Kathrin Breunig, Sandra Lange, Martina Ohme, Markus Hörer, Florian Sonntag, Andreas Schulze

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Recombinant adeno-associated virus (rAAV) is among the most widely used viral vectors for gene therapy. However, vector manufacturing remains a challenge as the choice of upstream production systems represents a trade-off between flexibility, scalability, and quality. Currently, adherent or suspension cell-based rAAV manufacturing processes are used by the industry. While different large-scale adherent production systems, such as the iCELLis® 500, are in use, there are several reasons to switch to suspension cell-based rAAV production processes. Suspension processes allow for better scalability (scale-up instead of scale-out) resulting in lower operational costs. Furthermore, suspension-based processes eliminate the need for animal-derived component supplementation. Resource- and labor-intensive approaches for process development and optimization, such as the use of shake flasks or bench-top bioreactors, limit the throughput required to efficiently improve rAAV production processes. Furthermore, these systems do not easily allow for changing of multiple parameters at a time (e.g., DoE - design of experiment) to determine the individual and combined effects of these changes on rAAV production. Therefore, high-throughput, automated microbioreactor systems represent ideal tools for effective bioprocess development and optimization. We previously presented that we successfully developed a microbioreactor system using the Ambr® 15 system that can be used as a suitable scale-down model for our suspension cell-based rAAV production processes. Furthermore, high throughput microbioreactor systems facilitate utilization of a DoE methodology to analyze multiple variables at a time, which enables optimization of rAAV production in our HEK293 suspension cell production platform. Here, we present the results of a DoE-based study evaluating the impact of process parameters, such as cell seeding density, total DNA per cell at transfection, and pH setpoint of the bioreactors on rAAV yield and quality. We applied a full-factorial central composite orthogonal (CCO) design allowing for quadratic modelling of vector yields as well as plasmid- and host cell-derived impurity packaging. These results allowed for the definition of key parameters and their set-points to enable efficient up-scaling of the rAAV production processes to larger-scale suspension manufacturing systems. Furthermore, the study provided insight into opportunities to optimize rAAV quantity and quality by adjusting these parameters. Overall, application of the DoE methodology to improve rAAV quantity and quality parameters allows for usage of the Ambr15® system as a superior tool for future manufacturing process development, optimization, and starting material development.

406 Gene Delivery from Flowable Linked Irregular Particle (FLIP) Scaffolds for Advanced Biologics Manufacturing

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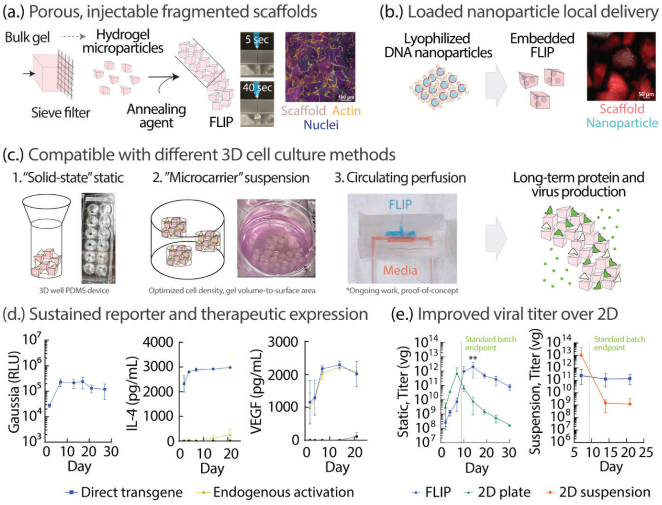
Purpose: Efficient gene delivery is essential for manufacturing therapeutic biologics, such as proteins and viruses. A key challenge has been high material costs and optimization to meet clinical demand. A possible way to improve production is hydrogel mediated gene transfer, in which nucleic acids are locally delivered via a loaded scaffold, acting as a reservoir for nanoparticles and providing sustained culture and expression. We developed "flowable linked irregular particle" or "FLIP" scaffolds, granular microporous hydrogels that overcome nanoparticle loading challenges and improve cell viability and transfection over time. Here, we demonstrate how FLIP can efficiently produce relevant biologics in a compact 3D environment and be adaptable to current bioreactor technologies. **Methods:** FLIP is prepared by embedding lyophilized polyanion-coated DNA/polyethylenimine nanoparticles within a bulk hydrogel, crosslinked using a degradable linker to allow timed release *[Fig, a-b]*. The gel is converted into fragmented microgels by controlled sieving. Cells are intermixed and secondary chemistry anneals the microgels into the porous FLIP scaffold. We explored several production approaches *[c]*: (1) static HEK293 culture in a FLIP "solid-state bioreactor", (2) suspension culture via FLIP "microcarriers," and (3) perfusion culture in a closed loop circulating system. Aside from reporter (Gaussia luciferase, GFP) and therapeutic (VEGF, IL-4) genes on a single plasmid, we assessed co-delivery of dCas9-VP64/sgRNA plasmids for endogenous protein expression. Last, we assessed triple plasmid delivery of AAV9 components (Helper, Rep/Cap, Transfer) for sustained virus secretion. For each method, we optimized the cell density and scaffold properties for enhanced expression and viability. Controls were either HEK293T plated in 2D tissue culture or HEK239F in suspension. Cells were cultured up to 30 days, with media sampled over time for protein (reporter assays, ELISA) or virus (qPCR, ELISA, MOI). **Results:** FLIP scaffolds previously promoted a sustained rate of reporter transfection, and this remained the case for therapeutic proteins from transgene and endogenous expression *[d]*. Both VEGF and IL-4 benefited from static FLIP culture, with no loss in rate of production compared to controls. For AAV, static FLIP gave sustained production over 2-3 weeks *[e]* before a significant loss in infectivity, while 2D plated cells declined after just one week in titer and quality. In suspension, FLIP production never overcame that of HEK293F, despite sustained titer and higher viability. Suspension

does afford easier scale up and compatibility with current bioreactors; however, the best quality AAV still came from static FLIP, with similar titer, infectivity, and capsid loading efficiency, but in a smaller footprint. Last, as proof-of-concept, we tested a modified solid-state FLIP using a perfusion fluidic device. From the circulating media, we saw improved cell viability and reporter expression compared to static FLIP. Ongoing work on long-term AAV production, scalability, and alternative materials will help to further establish this method. **Conclusions:** FLIP scaffolds offer a novel, more efficient approach to biologics manufacturing, with sustained culture giving better batch yields without loss in product quality, and the platform being easily adapted for specific products or mode of production.



407 Titer Boosting of HEK293-Based AAV Manufacturing Process Using Proprietary Small Molecule Additive and Successful Scale up to 200L

Jackson Leong

MTDC, Tenaya Therapeutics, South San Francisco, CA

Adeno-associated virus (AAV) is quickly becoming a safe and effective therapeutic modality for the delivery of potentially curative treatments for genetic diseases. One of the major challenges associated with AAV GT is to cost effectively produce AAV Drug Product (DP) with adequate levels of critical quality criteria (safety, identity, strength, purity, potency, and quality). Triple transient transfection using HEK293 process is widely used in both clinical and commercial scale manufacturing. The main limitation of HEK293 is scalability and viral productivity. Through the use of high-content screening leveraging deep knowledge of in the areas of cell biology, metabolic, anti-fibrotic, human genetics, tubulin and histone regulation, and extensive chemistry experience, Tenaya has developed a class of proprietary, novel, and selective small molecule boosters (SMB) that can significant increase AAV yield in cell line and cell culture media-independent manner. Additionally, SMB can enhance the scalability of HEK293-process by maintaining consistent yield up to 200L. And finally, SMB has demonstrated to have no impact on purity, quality, safety, and potency of the AAV viral vector and can be readily cleared in standard AAV purification process. This novel and selective class of SMB can potentially be transformational in debottlenecking AAV manufacturing and decrease of cost of AAV gene therapy.

408 Development of a Comprehensive and Risk-Based Viral Safety Assurance Strategy for the Manufacturing of AAV Gene Therapy

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The risk of viral contamination, arising from either the starting materials or the adventitious agents introduced during the manufacturing process, is an inherent feature in all biological products and can have serious consequences during clinical evaluation. Relative to biologics such as monoclonal antibodies or recombinant proteins, assuring the viral safety of gene therapy products is a complex process, as drug products are also viral vector particles. Therefore, having a comprehensive risk-based strategy to ensure viral safety, without impacting the identity, strength, quality, purity, and potency of the viral vector, is important to ensure patient safety. Tenaya Therapeutics, Inc. (Tenaya) has developed a consistent and scalable purification process for the production of adeno-associated virus (AAV) gene therapy made using Sf9/recombinant baculovirus for certain genetic heart diseases. Utilizing its purification process, Tenaya is able to demonstrate robust viral clearance, in terms of Log Reduction Values. This approach includes product- and process-relevant specific and non-specific model viruses and provides adequate assurance that AAV drug product is free of viral adventitious agents with appropriate level of safety margins.

409 Development of Cost-Effective and Scalable Recombinant Baculovirus Production Process for the Manufacturing of AAV

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After the early success of Zolgensma® and Luxturna®, adeno-associated virus (AAV) gene therapy is entering a mature stage and expanding its therapeutic potential to serve broader patient populations suffering from more prevalent genetic diseases. At Tenaya Therapeutics, Inc. (Tenaya), our Gene Therapy platform uses AAVs to deliver genes to specific cells in the heart to correct or compensate for functional defects. Genetic root causes of hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC) have the potential to be readily corrected *in vivo* using the delivery of AAVs. However, as AAV gene therapy moves into indication with greater prevalence, the feasibility of conventional triple transient HEK293 process as a manufacturing platform to produce sufficient quantities of drug product (DP), is limited by yield and scalability and is subject to high cost of goods (COGS). Tenaya has developed a proprietary manufacturing platform process using Sf9/recombinant baculovirus (rBV), which overcomes the limitations of yield, scalability and mitigates the high COGS. This Sf9/rBV manufacturing process includes a scale-independent recombinant baculovirus production process that consistently produces high quality recombinant baculovirus seedstock while maintaining productivity, quality, purity, safety, and potency of AAV DPs at the 1000L scale.

410 Seamless Scale-Up of Transient Transfection Process for rAAV Production from Shake Flask to Manufacturing-Scale Bioreactor

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Recombinantly produced adeno-associated viruses (AAV) are the predominant viral vectors used for in vivo gene therapies owing to their relatively good safety, long term transgene expression, ability to infect a broad range of cells and ease in achieving high titers at small-scale. One of the main challenges in bringing AAV-based therapies to patients, is the ability to efficiently translate bench-scale processes to commercial GMP manufacturing while meeting the high dosage requirements. In order to overcome this challenge, we have partnered with LogicBio® Therapeutics and Polyplus® for the development of a highly scalable AAV manufacturing platform with a capacity ranging from 1L to 200L and 2000L bioreactor scale. The vector used for the scale-up is a recombinant AAV8 coding for factor IX protein. The suspension process lasting 7 days is performed in batch mode in a stirred-tank bioreactor (STR) and includes a transfection step using the 2 plasmids-system mAAVRx™ technology (LogicBio) and FectoVIR®-AAV transfection reagent (Polyplus). First, some preliminary tests were conducted in shake flasks assessing parameters that are important for process scale-up, such as transfection mix preparation and transfer conditions. Next, the transfection process was translated at 1L scale into BioBLU® 1c Single-Use STR. A titer of $7.89x10^{11}$ vg/ml was obtained at this scale which was comparable to the titer obtained in shake flask using similar transfection conditions (see Figure 1). For the scale-up in STR, the chosen strategy was to maintain constant P/V (power input) and similar superficial gas velocity at all scales. As a first step of the scale-up, the AAV8 production process was successfully performed at 40L scale in an Eppendorf BioBLU® 50c Single-Use Bioreactor yielding a titer of $6.98x10^{11}$ vg/ml (see Figure 1). Following this, a scale-up of the process to 200L in a Pall Allegro 200L STR was performed. Consistent AAV8 titer (see Figure 1), cell growth, cell viability and metabolite profiles were obtained across scales, indicating a successful process scale-up to 200L. The next step is to scale-up the process in Pall Allegro 2000L STR. To sum up, in collaboration with LogicBio® Therapeutics and Polyplus®, Exothera has succeeded to seamlessly scale-up an AAV8 production process from shake flask to 200L scale reaching a titer of above $6x10^{11}$ vg/ml at all scales.

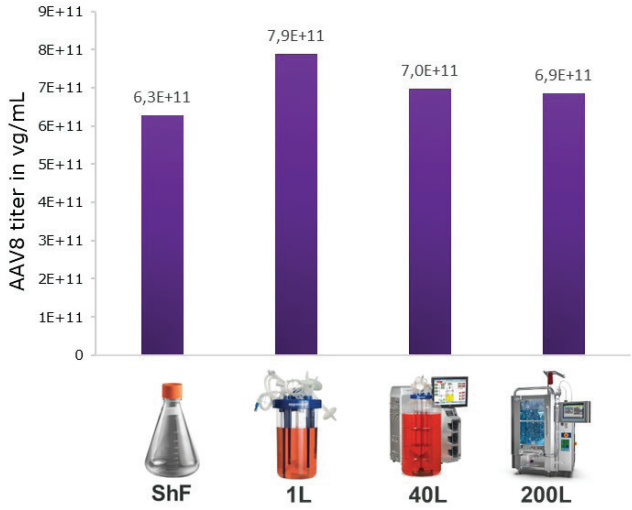

Figure 1: Genomic AAV titer measured by ddPCR on clarified lysate. n=1 at all scales.

411 Scaling-Up AAV- Manufacture GMP Production and Purification Using Innovative Plasmid-Free Technology

Bibek Ghosh[1], Yi-Hsin Fan[1], Ji Hyun Lee[1], YiYu Dong[1], Dominic Masciarelli[1], Alexander Lam[1], Honghu Quan[1], Annie Tran[1], Zhong Deng[1], Joseph Flounlacker[1], Ang-Chen Tsai[1], Bo-Jhih Guan[1], Heather Malicki[2], David Chang[3], Man-shiow Jiang[4]

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Adeno-associated viral vectors (AAV vectors) are essential tools for delivering gene therapies to patients, representing around 37% of the advanced therapies market. AAV vector is usually manufactured using plasmid-based transient transfection approaches. However, there are challenges with working with plasmids on a large scale, including cost, consistency and scalability. WuXi Advanced Therapies (WuXi ATU) developed TESSA™ (tetracycline-enabled self-silencing adenovirus) Technology for high physical titer and infectivity to meet the growing demand for AAV manufacture, such as increasing upstream productivities and overall recovery of downstream processes. Thus, it is crucial to develop upstream production and downstream purification strategies that high productivity, maintain yield, cost-effectiveness as well as high purity profiles. TESSA vectors directly encode the genetic material required for AAV production, removing the need for plasmids to perform that function. These vectors take advantage of the dual phases of the adenoviral lifecycle to produce AAV, while limiting adenoviral contamination. Early phase adenoviral gene expression provides help for high titer AAV manufacture, while late phase gene expression, responsible for adenovirus contamination, is switched off. This increases rAAV yield, packaging efficiency and compared with plasmid-based rAAV manufacture. The TESSA system includes co-infection with two adenovirus vectors: one adenovirus encoding a

gene of interest (GoI) and the other encoding AAV's replicative (*rep*) and capsid (*cap*) genes. The resulting physical and infectious AAV titers using TESSA technology were compared with values obtained using triple-plasmid transfection into WuXi ATU single clonal cell line from human embryonic kidney (HEK) 293 parental cells. Upstream production of AAV2 and AAV6 using the TESSA platform has been completed successfully in single use bioreactor. Both AAV6 and AAV2 are consistently produced using TESSA technology in scaling up single use bioreactors. The upstream GC titer of AAV2 is around 20-fold and AAV6 is around 10-fold higher with TESSA compared with triple plasmid transfection. TESSA technology presents a competitive solution for production of AAV, purified using AAVEX™ platforms to evaluate the yield and quality of final product. AAV2 and AAV6 both use a platform process using affinity column, scalable ion-exchange chromatography process. For AAV2, an overall recovery of 36% was achieved with 1.5E+14 total GC per L of upstream production. AUC analysis revealed that the final product obtained 88.2% of full capsids. AAV6 overall recovery of 52% was achieved with >5.0E+14 total GC per L of upstream production. AUC analysis revealed that the final product obtained 88.4% of full capsids. Taken together, WuXi ATU has successfully scaled up AAV vector production using TESSA technology with the product showing significant improvement on vector yield over plasmid-based methods. To ensure stable gene therapy production, the novel TESSA technology is integrated with intricate testing capabilities. This allows for in-house assay development, biosafety, viral clearance, and product release testing, reducing costs and increasing efficiency. Ultimately, this will accelerate the product approval timeline.

412 Global Strategy to Improve the Downstream Manufacturing Process of the Adeno-Associated Viral Vector for Glycogen Storage Disease Type Ia Treatment

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Ultragenyx, Woburn, MA

Although recombinant AAV (rAAV) vectors have been a common gene transfer vector in clinical development for greater than a decade, few of these products have been approved/authorized for commercial marketing. Acknowledging the need for a robust, productive manufacturing process, the chemistry, manufacturing, and control (CMC) strategy for a glycogen storage disease type Ia (GSDIa) specific rAAV production has experienced significant development and improvement. Here, we present the downstream process improvement from Process A to Process B for the purification of GSDIa-specific rAAV generated by HEK cell line. By the introduction of the harvest tangential flow filtration (HTFF) step prior to affinity chromatography, multiple aspects of the downstream process were improved, including the step yield of various unit operations and the global harmony of the downstream process, as shown in Table 1. Also, Process B generated a comparable drug substance to that of Process A, as shown in Table 2. Taken together, Process B was expected to enable a robust drug supply for GSDIa patients in Phase 3 clinical trials and beyond.

Table 1. Performance Improvement

Process Step		Process A	Process B	Improvement
HTFF	Product Concentration	N/A	20-fold Increase	HTFF concentrate AAV product concentration by 20-fold
	HCP Reduction	N/A	~50% Reduction	HTFF step gains ~70% HCP reduction prior to Affinity step
Affinity Chromatography	Column Volume (L)	1.0 X	0.2 X	Affinity resin COGS reduction by 5-fold
	GC Step Yield	~60%	~77%	Affinity GC step yield increase by >10% (absolute value)
AEX Chromatography	Column Volume (L)	1.0 X	0.2 X	AEX column COGS reduction by 5-fold
	GC Step Yield	~30%	~60%	AEX GC step yield increase by >30% (absolute value)
FTFF	Filter Size (m2)	1.0 X	0.2 X	FTFF filter COGS reduction by 5-fold
	Concentration Factor	400 X	80 X	FTFF concentration factor reduce by 5-fold

Table 2. Drug Substance Comparability

Quality Attribute	Process A	Process B
Genomic Concentration (GC/mL)	2.7E+13	3.5E+13
Full Capsid %	31%	37%
Residual AAVB Ligand (ng/mL)	<LOQ	<LOQ
Residual Benzonase (ng/mL)	<LOQ	<LOQ
HCP (ng/mL)	<LOQ	<LOQ
Aggregation %	2.3%	1.0%

413 Utility and Limitation of Density Gradient Analytical Ultracentrifugation for the Quantitative Characterization of Adeno-Associated Virus Vector

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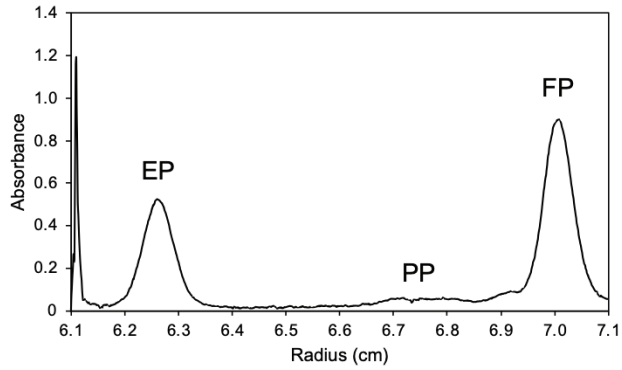
Introduction Adeno-associated virus (AAV) vectors are the leading platform for gene delivery of *in vivo* gene therapies. In the upstream process of AAV production, AAV vectors are produced as a mixture of the AAV particles with desired DNA (full particles, FPs), particles with incomplete DNA (partial particles, PPs) or without DNA (empty particles, EPs), and aggregates. Complete removal of impurities other than FPs is generally difficult during the following purification process. Meanwhile the presence of EPs may reduce transduction efficiency and increase the risk of immunogenicity. It is thus essential to assess the purity of AAV drug product more accurately. Sedimentation velocity analytical ultracentrifugation (SV-AUC) and band sedimentation AUC (BS-AUC) have been established that enable accurate quantification of FPs and EPs, as well as other components. However, both SV- and BS-AUC requires to be proficient in measurement and fitting the theoretical model to the experimental data, working several parameters to optimize the match between data and model. Other AUC method, density gradient AUC (DG-AUC), may applicable for AAV vector characterization. In ultracentrifugal field, the solution such as cesium chloride (CsCl) generates the density gradient, and macromolecule form a band where its own buoyant density equal to density of the solution at equilibrium. DG-AUC allows separated bands to be detected by absorbance optics with the multiwavelength (MW) detection. From information on the absorption properties of each band, it is possible to clarify the components contained in the AAV vectors. DG-AUC has also the advantage that one can analyze the obtained data using commercially available software or program, which might be important for the quality control of the virus vectors under cGMP compliant condition.
Results The measurement time was examined that takes for CsCl

density gradient reach at equilibrium in DG-AUC. AAVs with different serotype and different lengths of DNA showed similar time dependence. The characterization ability of DG-AUC was evaluated in comparison with that of SV-AUC. Compared with UV absorption properties of each component determined by MW-SV-AUC, observed peaks in MW-DG-AUC were assigned as shown in the figure. Then we examined the quantification of the ratio of FP and EP (F/E ratio) by DG-AUC. The linear correlation was confirmed for F/E ratio between DG- and SV-AUC and indicated that DG-AUC is a quantitative analytical method equivalent to SV-AUC. Finally, we established the peak assignment flowchart for MW-DG-AUC, then the obtained result was compared that of MW-BS-AUC using AAVs with different DNA lengths. **Conclusion** Using the flowchart, accurate evaluation by DG-AUC tends to difficult compared to SV- or BS-AUC especially for AAVs containing minor components such as PPs or aggregates which leads to undetectable or overlapping peaks. Therefore, one should use SV- or BS-AUC for the more accurate and reliable evaluation of size distribution of AAV vectors for gene therapy. Nevertheless, DG-AUC can also applicable to characterization of any AAV vectors using optimum conditions. In addition, DG-AUC is the only analytical method to evaluate the particle density heterogeneity that cannot be detected by other orthogonal methods. SV- or BS-AUC and DG-AUC are therefore complemental methods to each other for the reliable purity assessment of AAV vectors.



414 Belief BioMed - High Yield rAAV Production Platform with Accurate Analytical Assay

Zengmin Du[1], Wei Jiang[1], Philip C. Sin[2], Jane Zheng[1], Xiao Xiao[1]

[1]Belief BioMed Limited, Shanghai, China,[2]Belief BioMed Limited, Hong Kong, China

Introduction: HEK293 cell and transient transfection method is the most versatile platform for packaging rAAV. However, HEK293 cell platform has been hampered by low yield and high incidence of empty virus particle. Belief BioMed has screened a clonal HEK293 suspension cell line, which has been identified as the optimal rAAV production platform, enabling high-titer rAAV production for multiple serotypes with low empty virus particle (<50%). Here, we present an overview of the current rAAV production platform in Belief BioMed, focusing on performance of the upstream and downstream unit operations in 200L and 500L scale, as well as virus genome integrity, purity and key residual impurity level of rAAV vectors.Conclusion: Process for rAAV manufacture have evolved significantly with the boom of gene therapy field. Product yield has improved to over 1e15vg/L, which will effectively reduce the cost of gene therapy product. From the current data, product A and product B show high yield and consistent quality under the viral vector manufacturing process of Belief BioMed, which demonstrates a linear scalability up to 500L. Additionally, both product A and product B have been applied to clinical trials in China and show high safety and drug efficacy. To further support gene therapy development, future studies are designed to improve the cell density during transfection process and the infectivity of viral vectors.

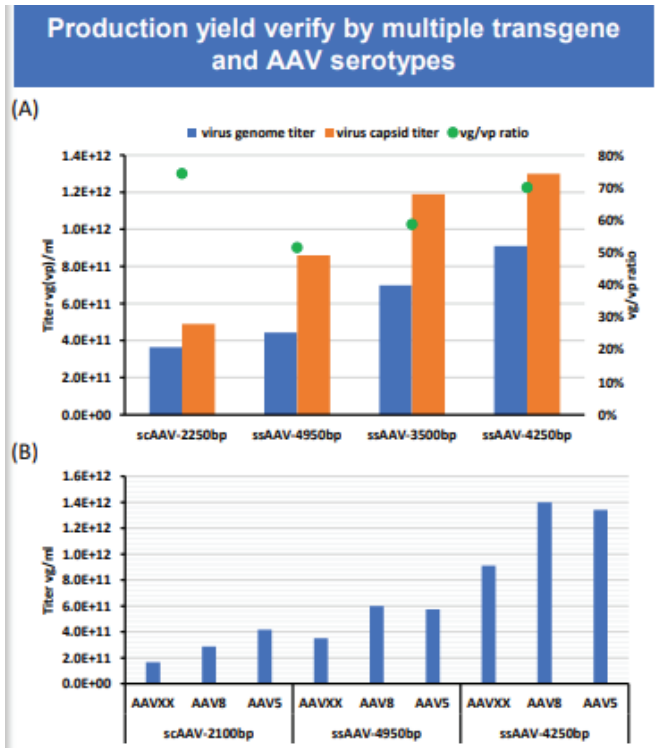


Figure 1. (A) HEK293 cell production yield and vg/vp ratio identification by different transgene. (B) HEK293 cell production yield identification by different AAV serotype. All the data were based on shaker flask culture and vector genome titer were determined by ddPCR

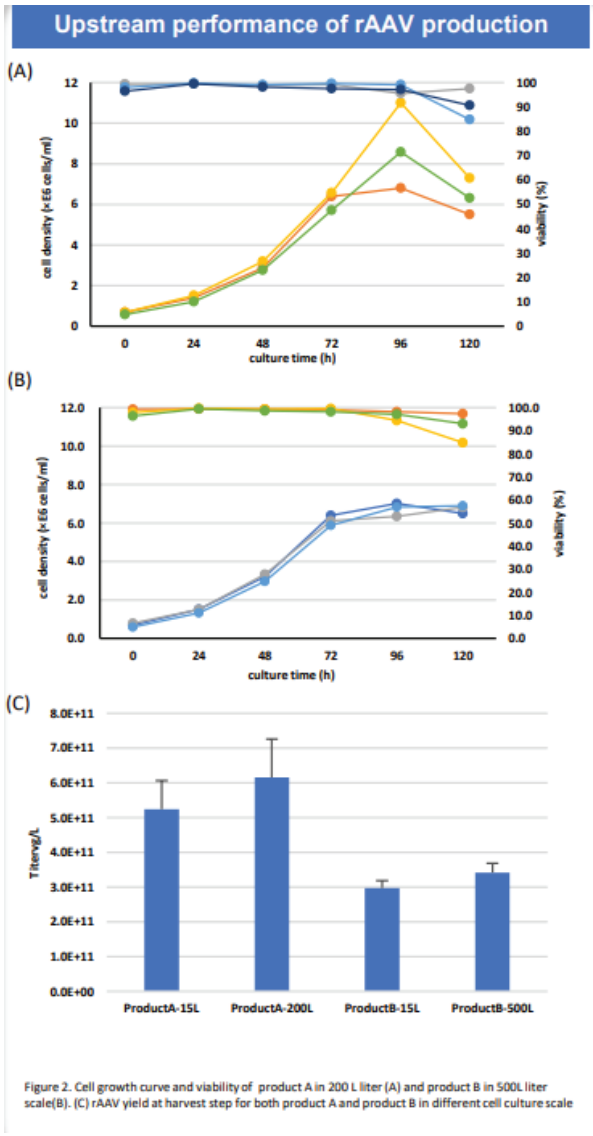


Figure 2. Cell growth curve and viability of product A in 200 L liter (A) and product B in 500L liter scale(B). (C) rAAV yield at harvest step for both product A and product B in different cell culture scale

415 Early Phase Viral Clearance Study Design for AAV Manufacturing Process

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Viral safety is a critical component of the quality evaluation of biotherapeutics to avoid any adverse impact on patients. Viral safety strategy revolves around 3 key pillars: prevention, detection, and removal. This presentation will focus on the final point, removal. Developing a downstream purification process capable of reducing adventitious viruses is critical to ensure patient safety. By performing meticulous, phase-appropriate, viral clearance (VC) studies these removal and inactivation techniques can be tested. It is important that viral safety and process development teams are implementing and continuously improving viral clearance strategies. One of the many challenges that come with designing a viral clearance study is ensuring what is done in controlled labs will be representative of what is happening at production scale. Here, we will be presenting an approach for designing VC studies including scale-down model verification and early phase study design.

416 A New Method for AAV Purification Bypassing Diafiltration, Depth Filtration, and Buffer Exchange

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Purpose: The Typical workflow of AAV purification involves lysis step, concentration (depth filtration/TFF) and column chromatography. The process of depth/diafiltration is laborious, time consuming and expensive. The purpose of this new method is to concentrate lysate and media containing AAV particles without the use of diafiltration and remove unrelated contaminants and cell debris usually eliminated by depth filtration technique. The method also eliminates the need for buffer exchange machine and can make the AAV containing media/lysate ready for FPLC purification. **Methods:** AAV particles were produced in HEK293 suspension cells using the triple transfection plasmid method. Cellular lysates were treated with Benzonase for 1hr at 37°C following by centrifugation for 30 minutes. Total lysate + media were allowed to mix with AAVX beads overnight at 4°C. On the following day, beads were pelleted by centrifugation at RT. Unbound material was removed. Beads were resuspended in lysis buffer for the AAVX and added to small columns. AAV particles were eluted using elution buffer. Samples were dialyzed overnight in 1X PBS containing 0.01% PF-68. Samples were collected and sterile filtered and were subjected to FPLC. The purity of the virus was verified using western blot and as well as Coomassie Blue staining. **Results:** Our platform approach could be applied to different serotypes including AAV2, AAV8, and AAV9. Western blot analysis detected VPs (VP1, 2, and 3) in eluted samples and gel staining shows a significant elimination of non-VPs proteins. The FPLC analysis shows the characteristics peaks for AAV, and the purified samples yielded characteristic protein bands. **Conclusions:** The new purification method using Affinity-bead was successful in eliminating the need for diafiltration and other concentrating methods as well as buffer exchange. This method is cost-effective, scalable, and timesaving for production of different AAV serotypes.

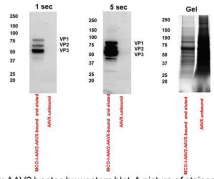

Fig.1. Detection of VPs in eluted samples from AAV2 lysates by western blot. A picture of stained gel is shown on right. VP1, VP2, and VP3 are shown in first lane in eluted fraction. In contrast, unbound fraction showed no VPs. Stained gel confirmed the removal of majority of nonspecific AAV particles (2 lane in left picture).

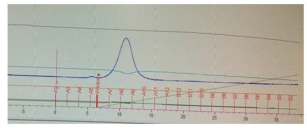

Fig.2. Detection of AAV2 particles and elution from FPLC.

417 Intensified Production Process for Gorilla Adenoviral Vector, GRAd: Yield, Purity, Potency and Stability Results

Stefano Colloca, Adriano Leuzzi, Alessia Noto, Giuseppina Miselli, Eleonora Lilli, Alessandro Moretti, Andrea Canosa, Flavia Cenesi, Michela Gentile, Romina Scala, Stefania Capone, Antonella Folgori, Leonardo Sibilio, Federico Napolitano, Fabiana Grazioli, Virginia Ammendola

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Introduction: There is a need for viral vectors for vaccine and advanced therapy applications with low seroprevalence in the human population. Simian adenoviruses derived from the chimpanzee, bonobo, and gorilla, are not known to infect or cause pathological illness in humans, and consequently have low/no seroprevalence (0% - 18%) in the human population. Adenoviral vectors derived from simian adenovirus and more specifically from great apes have been studied in thousands of human subjects using vaccine vectors encoding different antigens (relevant to Ebola, malaria, hepatitis C, human immunodeficiency virus [HIV], and respiratory syncytial virus [RSV]). It is well established that this vaccine platform is safe and can generate potent, durable, and high-quality T cell and antibody (Ab) responses. Impact: ReiThera developed a Covid-19 vaccine, based on a novel gorilla adenovirus classified into species C which was isolated from a captive animal and converted into a replication-defective adenoviral vector generating GRAd32 COV2 vector. GRAd32 COV2 vaccine has completed the phase II clinical trial in 2021 showing a well-tolerated safety profile and strong immunogenicity. Methods: In parallel ReiThera has developed a production process based on the proprietary high yield packaging cell line ReiCell 35S based on low passage HEK293 cells. The process was designed to provide high volumetric yield by exploiting perfusion and high cell density at infection in single-use stirred tank bioreactor. The downstream process was based on selective host cell DNA precipitation step followed by membrane chromatography, polishing step and formulation by TFF. Results: The analytical results demonstrated that the process is able to provide a very high yield with the final product meeting all specifications in terms of potency and purity. Importantly, the final product was demonstrated to be stable at +4°C for more than one year. **The process was initially set up in 2L bioreactor and thereafter scaled up to 1000-L and 2000-L single-use STR.** Conclusions: **The technology for viral-vector production is validated. The technology can be used for other vector types, like AAV, especially if a systemic gene therapy requires a large amount of vector production.**

418 Highly Intensified and Low-Cost Manufacturing of Viral Vectors Using HIP-Vax® Technology

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The field of virotherapy is rapidly transitioning from early-stage development to clinical efficacy testing and commercial approval. Recent approval of three cell and gene therapy products for beta thalassemia, cerebral adrenoleukodystrophy, and hemophilia B are widely expected to only be the start. Viral vectors are commonly used in virotherapy applications due to their natural ability to infect a broad variety of cells and express genes of interest for extended periods on time or integrate in the host-cell genome and correct or inactivate faulty genes. Although major milestones in clinical outcome of these therapies have been reached, the manufacturing has been met with significant challenges. Therefore, the global roll-out of these novel, life-saving therapies for diseases previously thought uncurable, is held back. Here, we describe the utilization of our HIP-Vax® platform that was developed for manufacturing of viral vaccines with ultralow costs for the production of viral vectors. Through successful process and analytical development, we have been able to produce high titers of viral vectors through several methods in one manufacturing platform, including infection-, transfection-, and induction-based production processes. All methods produced equivalent titers in bioreactor as they did in standard culture flasks, indicating a high compatibility of both traditional as well as next generation manufacturing methods with our HIP-vax® platform. Downstream processing yielded high titer, highly purified drug substance batches for preclinical and clinical evaluation. Finally, our HIP-vax® platform is readily scalable from small scale R&D and early stage clinical trial productions to late stage and commercial scale manufacturing, which will increase efficiency towards licensure. In summary, the HIP-Vax® platform is a valuable addition to the existing manufacturing methods, enabling the delivery of these desired therapies worldwide.

420 AAV8 Capsid Stability Dependence on Mg2+ Ions

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As a vector for gene therapy, the adeno associated virus (AAV) family has enjoyed notable success and popularity as of late. These viral capsids undergo tremendous physical stress during production and purification processes, leading to loss of product. Having observed increased on-column stability of adeno associated virus serotype-8 capsids when supplemented with Mg^{2+}, we used monolithic anion exchange chromatography and ddPCR/ELISA based analytical methods to further explore this relationship during column processes. As we demonstrate this effect to be particularly pronounced in empty capsids, we also report its impact on an intentionally empty-only capsid production of AAV8. We finally show that susceptibility to stress increases in the absence of Mg^{2+} ions,

an effect that can be exacerbated by use of a chelating agent, EDTA. The results suggest that the supplementation of chromatography buffers with Mg^{2+} improves yields, as well as the homogeneity of chromatographic species when separated by ion exchange methods. Beyond the reduction in cost of production, this would imply that Mg^{2+} is a necessary component in any effort to enrich full capsids.

421 Step Gradient Development for Full Particle Enrichment of AAV on Anion-Exchange Monoliths is Hindered by Inconsistent Column Lots

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Empty capsids are by-products of adeno-associated virus (AAV) production and are generally accepted as undesirable in drug product preparations due to complications surrounding immunogenicity and dose-related toxicity. Recent proposed draft guidance for Food and Drug Administration (FDA) consideration recommends a maximum release criterion of less than or equal to 30% empty capsids in AAV-based gene therapy products. Efficient control or removal of empty capsids through optimization of vector genome encapsidation during upstream production or through physical separation by downstream unit operations is thus a critical consideration for gene therapy manufacturers. To remove this product-related impurity, downstream purification strategies often aim to exploit the subtle difference in isoelectric point (pI) between full and empty AAV particles through ion-exchange chromatography (IEX). As many AAV serotypes have acidic pI values, anion-exchange (AEX) columns are typically selected as the preferred stationary phase for chromatography as the AAV charge state is maximized in high pH buffer solutions. AEX monoliths (e.g., CIMmultus QA by Sartorius) have emerged as a popular modality for on-column separation of full and empty AAV as they possess several attractive properties, including flow rate independent performance (i.e., binding is not diffusion limited) and large flow-through channels (i.e., well suited for separation of large biomolecules). Indeed, we previously developed a chromatographic method using an anion-exchange monolith to enrich full AAV particles to greater than 90% in drug product preparations. This method employed a shallow linear gradient of salt to elute the loosely bound empty AAV particles first, followed by the more tightly bound full AAV particles at higher ionic strength. While successful at full particle enrichment, this method could be improved for commercial bioprocessing by conversion to a dual step gradient method, wherein a first wash step at a given concentration of salt elutes the empty particles and a second wash step at a higher salt concentration elutes the full particles. Such a method is easier to execute at scale, promising less user interaction, less buffer consumption, and less eluate volume. In this study, we aimed to develop a dual step gradient method on the AEX monolith for full particle enrichment. This work involved screening buffer solutions to identify the ideal salt concentration of the first wash step for maximum elution of empty particles, without elution of full particles, and to identify the minimum salt concentration of the second wash step for maximum elution of full particles. Once these conditions were determined, we then characterized the process robustness, including vector genome recovery and enrichment across runs of various load materials, buffer lots, and columns lots. We found that while the method was at times capable of high vector genome recovery (>90%) and full particle enrichment (>90% full particles), lot-to-lot inconsistencies in the monolith columns often lead to subpar or unpredictable run performance, including significant loss of full particles during the first wash step (e.g., 30% of full particles) due to premature elution. Based on these results and without further improvements to the technology, we do not recommend step gradient development on the monolith columns and instead advise to scale up using a linear gradient method. For sake of comparison, we also successfully developed a dual step gradient method for a resin-based AEX system which showed consistent separation performance regardless of column lot. This research was funded by Sarepta Therapeutics.

422 Next-Generation Rep-Cap Dual Baculoviruses for AAV Production in Insect Cells

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uniQure's AAV-based gene therapy manufacturing platform is based on an insect-cell baculovirus expression system, where infectious baculoviruses deliver the different components needed for AAV production. uniQure's first-generation platform consists of an infection with three separate baculoviruses each containing AAV-Rep, AAV-Cap and the therapeutic cassette. uniQure's next-generation platform is based on a dual baculovirus infection, one containing both AAV-Rep and AAV-Cap (DuoBacs) and the second one containing the transgene. Several Rep designs were evaluated in combination with a representative AAV capsid. These DuoBacs showed improved genetic stability within the baculovirus genome. AAVs produced using these DuoBacs were assessed for several quality parameters in both small and large scale productions. A few of these designs showed improved yield, empty to full capsid ratios, DNA packaging and potency. These designs need to be further investigated in order to assess the in vivo biodistribution and potency.

423 CMC Strategies for AAV Gene Therapies for Highly Prevalent Diseases

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Biological research applications and potential treatments for genetic and prevalent diseases are now being made possible by gene therapy. At present, viruses are the best tools for transferring genes into cells. Recombinant adeno-associated viruses (rAAVs) have become the most popular vectors of choice for many therapeutic and fundamental applications. Approved products and the number of clinical trials

using rAAV are continuously increasing, demonstrating the necessity of standardizing (and improving) the manufacturing, and purification of these vectors to meet the quantitative and qualitative requirements of regulatory agencies. One of the most important factors to move the gene therapy field forward is the Chemistry, Manufacturing, and Controls (CMC), which is responsible for all the activities related to process purity. In this study, we focused on improving our rAAV production system in insect cells (Sf9), which is based on the baculovirus expression vector (BEV) that uses the Bac-to-Bac baculovirus expression system. We modified the selection markers of the donor plasmid (pFastBac) in the Bac-to-Bac system by removing the Gentamicin resistance gene and adding an extra positive selection marker (rpsl) to remove the donor plasmid from recombinant Bacmid preparations. We also evaluated the BEV stability and yield level of rAAV during successive passages of the BEV (P0 vs P2) in insect cells. Our result demonstrated that the modified donor plasmid (pFastBac) leads to enhanced recombinant Bacmid quality and decreased residual encapsulated DNA titer. This represents an important improvement in the rAAV production system which should assist with improved vector preparations for research and clinical applications including prevalent indications with higher overall product demand.Biological research applications and potential treatments for genetic and prevalent diseases are now being made possible by gene therapy. At present, viruses are the best tools for transferring genes into cells. Recombinant adeno-associated viruses (rAAVs) have become the most popular vectors of choice for many therapeutic and fundamental applications. Approved products and the number of clinical trials using rAAV are continuously increasing, demonstrating the necessity of standardizing (and improving) the manufacturing, and purification of these vectors to meet the quantitative and qualitative requirements of regulatory agencies. One of the most important factors to move the gene therapy field forward is the Chemistry, Manufacturing, and Controls (CMC), which is responsible for all the activities related to process purity. In this study, we focused on improving our rAAV production system in insect cells (Sf9), which is based on the baculovirus expression vector (BEV) that uses the Bac-to-Bac baculovirus expression system. We modified the selection markers of the donor plasmid (pFastBac) in the Bac-to-Bac system by removing the Gentamicin resistance gene and adding an extra positive selection marker (rpsl) to remove the donor plasmid from recombinant Bacmid preparations. We also evaluated the BEV stability and yield level of rAAV during successive passages of the BEV (P0 vs P2) in insect cells. Our result demonstrated that the modified donor plasmid (pFastBac) leads to enhanced recombinant Bacmid quality and decreased residual encapsulated DNA titer. This represents an important improvement in the rAAV production system which should assist with improved vector preparations for research and clinical applications including prevalent indications with higher overall product demand.

424 Upstream Optimization Paved a Straightforward Path for High Percentage of Full Capsids in AAV6

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Adeno-Associated viruses (AAV) are a potent tool in the expanding field of cell and gene therapy as they offer low immunogenicity and can facilitate gene transfer or gene editing for *in vivo* or *in vitro* applications via the efficient delivery of genetic material. However, the AAV potency and safety are highly dependent on the genome packaging efficiency, including full particle percentage among the total particles, the distribution of partial particles, and the amount of empty particles. Because clinical applications for AAV are increasing rapidly, there is a high demand for further increasing yields as well as product quality, particularly for a higher percentage of full capsids in the final product. One of the biggest challenges in current AAV manufacturing is to achieve >70% full particles in the final drug substance/drug product (DS/DP). The percentage of full particles is influenced by several factors including the low percentage of full particles produced from upstream processes, the separation and enrichment processes from downstream purification, etc. Here, we describe a method of upstream optimization to achieve high full particle enrichment (>70% full) in final purified AAV products without further development of the downstream process. A platform process for AAV6 production has been well-established and is based on transient transfection of serum-free cells grown in suspension. In this process, we have evaluated multiple parameters in the upstream production process (for example, cell growth, transfection reagents, DNA concentration, etc) to reach titers > 1e11 vg/mL and >15% full particles based on dynamic light scattering (DLS) using Design of Experiment (DoE) studies in shake flasks. Furthermore, our data demonstrated good scalability from shake flask to a 2L stirred tank reactor (STR) in terms of genome titer and full%. The challenge for the baseline process was that final AAV could only reach ~50% full particles even after IEX enrichment. To avoid changes in the downstream process and accelerate the development timeline for this project, a follow-up DoE was performed to optimize the ratio among 3 transfection plasmids to increase AAV full percentage. Batch purification was used to determine upstream processes with higher percent full particle via DLS, and the top 4 conditions achieved >1.5e11vg/mL and 17-23% full (DLS). Then, the top 2 processes were scaled up into 2L STR and particles were purified through our platform affinity chromatography using AKTA for further verification. The data showed the affinity purified AAVs from both processes were > 1e13 vg/mL and 30-40% full (via Analytical Ultracentrifugation [AUC]). Since the GMP friendly downstream process usually include Ion Exchange (IEX) chromatography after the affinity column to enrich the full particles, these 2 upstream processes have been also applied through IEX separation and our data demonstrated the final AAV titer were at 1e13vg/mL level and most importantly, the full particles were enriched to >70% full. Aiming for offering a scalable and GMP compatible process in a fast timeline manner, the DoE study for upstream optimization provided

a straightforward path to achieve higher genome titer and quality specification (>70% full particles) to meet clinical requirement without making any changes in downstream unit operations.

425 Development and Scale-Up Validation of Small Molecule Enhancers for Increased Viral Vector Yield

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At-scale manufacturing of gene and cell therapy vectors is a complex process, often resulting in insufficient manufacturing yields to satisfy developmental and commercial demand. While the last decade has seen major advances in the viral platform, bioreactor design, cell line and media formulation optimization, the industry still faces major challenges with production consistency and yield. An often overlooked and underappreciated bottleneck in cell culture-based manufacturing is the presence of cellular innate antiviral immune pathways that remain partially, if not fully, intact in producer cells. These well-conserved pathways are triggered via the detection of foreign nucleic acid and/or molecular bi-products of viral replication/assembly resulting in blunted viral vector yields in both infection and transfection-based manufacturing strategies.Virica's Viral Sensitizers (VSEs™) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently dampening cellular antiviral defenses. Leveraging high-throughput methods, Virica has assembled a library of over 130 small molecules which enhance viral production by transiently antagonizing a broad range of cellular innate antiviral pathways. Owing to different molecular mechanisms, VSEs™ can be combined and formulated for specific uses across various viral and cellular platforms.Here we describe the development of custom small molecule formulations for enhanced production of therapeutically relevant vectors MVA and AAV, using high-throughput methodologies. Furthermore, we present validation data supporting the scalability of these custom formulations in formats representative of large-scale commercial production platforms (iCELLis Nano for MVA; suspension bioreactor for AAV). Ultimately, this data supports the use of small molecule enhancers, such as Virica's VSEs™, for increased production of viral vectors at scale.

426 Optimizing Shelf Life of AAV8 and AAV9: DP Container Closure Selection for Storage and Shipping

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Rubber stoppered glass vials are a popular primary container closure system for storing and delivering liquid formulations due to their low leachable levels and minimal retention of most drug products. More recently, polymeric vials have gained increasing prevalence due to a more rugged construction and lower cost. With the emergence of adeno-associated viruses (AAVs) as a leading drug platform for gene therapy and the development of new material constructions for container closure systems, the determination of optimal containers with the least impact on product stability is critical for determining storage and shipping conditions as well as maximizing shelf life. Two of the most common serotypes of adeno-associated virus vectors (AAV8 and AAV9) were formulated using a model Tris-HCl buffer and were subjected to storage in four of the most commonly used container closure systems on the market. To evaluate the impact of container closure on product stability, vials were placed under stress conditions meant to mimic real-world handling or conditions used to project long-term stability. The conditions evaluated included extended holds under expected clinical storage temperatures; short-term holds under thermal stress conditions often used to predict shelf life; and other stresses meant to mimic typical shipping and handling, such as shaking, storage on dry ice, and freeze thaw cycling. Product quality was evaluated using a variety of methods to determine changes in concentration, titer, aggregates, empty to full ratio, thermal stability, subvisible particles, infectivity, and turbidity. The findings from this study provide an initial roadmap for gene therapy companies, aiding in the selection of container closure components during drug product development and commercialization.

428 Finding Flexibility in a Templated Vector Manufacturing Process - Understanding Process Efficiencies in the Downstream Purification of a Model AAV Product

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Templated viral vector manufacturing processes can provide a distinct advantage by reducing overall process development time, hastening delivery of novel therapeutics to the clinic, and ultimately providing improved patient outcomes. However, a templated process must also be flexible enough to accommodate individual vector and transgene combinations that can impact vector production (e.g., variable titers) in the bioreactor and alter harvest composition. These conditions further affect downstream purification causing processing bottlenecks and increasing costs unnecessarily. Here we describe work using model AAV2 in a downstream vector manufacturing process comparing alternative approaches to increase processing efficiencies (including time and cost). Specifically, we focus on improving affinity chromatographic performance by comparing direct loading of clarified lysate to clarified lysate concentrated using tangential flow filtration (TFF) by quantifying changes in the dynamic binding capacity (DBC) of the resin under otherwise identical loading conditions, such as retention time and column volume. Our findings demonstrate areas where we can take advantage of increased loading onto affinity chromatographic resin that accompanies properly TFF-concentrated feed. We identified conditions that resulted in a greater than 4-fold increase in the measured DBC, providing commensurate reduction in both the volume of resin required to process an equal load of clarified lysate as well as the time required to load the material. However, we also model circumstance in which sufficient titer from the bioreactor mitigates the potential benefit of feed concentration and where direct loading of clarified harvest unto the chromatographic resin is more beneficial. Our findings demonstrate the importance of identifying

areas within a viral vector manufacturing template where a deeper understanding of the holistic process can provide flexibility to support diverse products while maintaining development speed necessary to deliver life-changing therapies where they are needed.

429 Optimized AEX Buffer Formulations for AAV Full Capsid Enrichment

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The developed tropism of Adeno-Associated Viruses (AAV) allows them to target human-specific tissues, conferring AAV therapeutics with the ability to become primary vectors for gene delivery. However, obtaining viral titers required to produce the desired therapeutic effect can be challenging due to inherent product related impurities, such as empty capsids. Towards this end, improvement of the Anion Exchange chromatography-based purification (AEX) step has been a major focus in the field. We have employed an extensive design of experiments (DOE) for AAV full capsid enrichment, utilizing multiple process parameters (including pH, numerous elution salts, excipients, surfactants, stabilizers, and osmolytes). In our studies, we have identified a set of serotype-specific buffer formulations that have achieved the required quality attributes of empty capsid removal while maintaining maximum recovery and infectivity of the viruses. A unique feature of these buffer sets is that they provide multiple formulation compositions, allowing selection of the top candidate based on raw material needs, grade, cost, and supply access. These properties afford greater flexibility to scale up manufacturing processes while ensuring high quality therapeutic products.

430 Development and Qualification of a Multiplexed ddPCR Assay to Evaluate DNA Integrity

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Droplet digital PCR (ddPCR) has become a robust and effective method to obtain the titer of gene therapy products. Although ddPCR for the gene of interest (GOI) can effectively quantify the gene product, it does not give information on the integrity of the packaged DNA, which is important for product characterization and process development. A number of genetic approaches are available to assess DNA integrity but often introduce bias or provide partial information. Furthermore, only a few of these methods have been fully qualified. This abstract discusses the advantages of Multiplex ddPCR as a measure of AAV packaged DNA integrity and describes the development and qualification of the method. In principle, Multiplex DNA leverages the use two primer sets, each at opposite ends of the gene of interest. The assay then determines the % of the packaged DNA that has both primer sites intact and thus the full length of the GOI. The presence of only one or the other primer sites is indicative of a truncated or fragmented GOI. Method development was performed and illustrates that AAV capsid could be diluted enough to be separated into the individual droplets to allow for a clear quantification of the individual viral particles. Further development included optimization for sample preparation and dilution which can substantially impact the accuracy of the integrity value. Qualification testing for the Multiplex ddPCR assay included assessment of linearity, accuracy, intermediate precision/repeatability, and specificity. In addition, primer sets across the GOI indicate that strand breaks occur across the gene of interest and not at a specific "hot spot". Through development and Qualification of Multiplex ddPCR, we have shown that the method can quantify the intact gene of interest in a robust, reproducible and precise manner. The qualified method can be used to support process development, product characterization and AAV stability assessments.

431 Prediction of Adeno-Associated Virus Manufacturability by Machine Learning

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Gene therapy is transforming human health by addressing rare diseases with single dose-treatments of therapeutic adeno-associated viral (AAV) vectors. However, the exorbitant financial cost of these treatments is a key roadblock for the widespread adoption of gene therapies. Manufacturing cost is a major contributor to Cost of Goods Sold (COGS) for genomic medicines. The lowering cost of high-throughput DNA synthesis, recent advancements in artificial intelligence and increasing computing power have enabled the application of machine learning (ML) to improve manufacturability of AAV capsids. We develop a state-of-the art ML model to predict the manufacturability of AAV capsid mutants based on the amino acid sequence of the capsid monomer, viral capsid protein 1 (VP1). Our model is trained on publicly available data of capsid fitness of AAV2 VP1 single point mutants. First, we use a protein language model (UniRep) to embed mutant protein sequences into biologically meaningful numerical vector representations. Then, we train and evaluate several classical ML regression models to predict the fitness of the mutants. We augmented a Random Forest model trained upon UniRep embeddings with a Ridge regression model trained upon one-hot embeddings that further improved the prediction accuracy of the model. Encouragingly, we achieved a significantly high prediction power (Pearson correlation = 0.82) on the held-out test set using our predictions from the generated UniRep+One-Hot model. Finally, we validate our model on two independent datasets not used for model training. In the first dataset, we show that the model can differentiate between capsid forming and non-forming sequences by their predicted fitness scores (P-value: 0.042). For the second dataset, we achieved high correlation between the model-predicted and the measured fitness score (Pearson correlation =.692). In the future, this model could be used as an *in-silico* screen for AAV capsids to reduce the time necessary for *in-vitro* experiments. With this work, we aim to establish an ML-based platform to increase manufacturability of clinical AAV capsids to make AAV gene therapies economically viable for patients.

432 Development of a Platform for Parallel Functional Evaluation of Cell Type Specific Synthetic Promoters for Gene Therapy

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One of the key components adeno-associated viruses (AAVs) is a promoter that drives transgene expression. Optimal promoters are cell type-specific to avoid toxicity, compact to fit into AAV, and drive a precise level of expression. Unfortunately, identifying and functionally testing promoters is laborious and time consuming. To that end, we built a system to screen a library of synthetic promoters and read out specificity and expression levels for each promoter on a single-cell level and in a single experiment. Such a screen allows for rapid selection of cell type-specific promoters from a large number of candidates. Moreover, it can deliver a set of cell type-specific and compact promoters that drive a range of expression levels, allowing for precise control of transgene expression. We created an AAV plasmid with a modified 3' UTR in the transgene cassette that contains a variable DNA barcode sequence compatible with single cell sequencing approaches. This vector served as a backbone to produce an AAV library, where each member contains a different synthetic promoter sequence coupled with a unique barcode. We built and optimized this system via testing AAVs individually and as a library in mouse utricle explants, using immunohistochemistry to evaluate levels of a GFP reporter and to compare against single-cell RNAseq (scRNAseq) detection of the promoter barcode.We used previously internally characterized promoters to build and optimize our technology. Our scRNAseq pipeline detected barcodes for each promoter in our pools and enabled us to read out specificity and expression levels across the cell types and cell states *ex vivo* and *in vivo* in mouse inner ear. We compared the scRNA-seq results to immunohistochemistry data measuring GFP reporter expression and found that the results were consistent in matched single-vector control samples. Following this work, we applied our system to completely novel inner ear promoters and characterized their specificity and expression levels. Our approach allows for fast and efficient synthetic promoter screening to deliver promoters for gene therapy that are compact and drive optimal expression levels of the transgene. This method should allow us to accelerate development and testing of novel gene therapy tools in the inner ear.

433 Mapping Administration Route-Dependent Transduction Profiles of Commonly Used AAV Vectors in Mice by Multiplexed Barcode Sequencing

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AAVs are important gene therapy vectors and attractive research tools to modulate gene expression in various tissues of interest. Both preclinically and clinically, the biodistribution and thus transduction profiles of AAV variants heavily depend on the administration route. Yet, despite the plethora of available capsids and the continuous selection of novel improved capsid-engineered variants, a systematic evaluation of the administration route dependency of AAV tropism has been lacking so far. Here, we established the transduction profiles of a unique collection of 34 well-known AAV capsid variants (wild types and published engineered capsids) for three administration routes, namely intravenous (i.v.), intraperitoneal (i.p.) and intratracheal (i.t.) injection, in C57BL/6 mice by state-of-the-art multiplexed biodistribution analyses based on AAV genome-barcoding. To that end, the AAV library ($1.36x10^{12}$ vector genomes per animal) was injected into eight animals per administration route, and DNA and RNA were isolated from all major organs 14 days later. Vector genomes (DNA) and transcripts (RNA) were then quantified by next-generation amplicon-sequencing. As expected, the analysis confirmed a pronounced liver transduction by most AAV variants after i.v. administration, as well as known transduction patterns for benchmark capsids, for example AAV-PHP.eB (brain) and AAV2-ESGHGYF (lung). In contrast, i.p. administration decreased the liver tropism of many vectors and led to capsid-specific changes in overall biodistribution. While some variants showed prominent transduction of the diaphragm, for other AAVs, broader distribution was observed, as evident from enhanced expression in abdominal and thoracic tissues. To further validate these results, the biodistribution profiles of selected AAV library variants were individually assessed in follow-up studies via qPCR and histology. For local delivery to the lung by i.t. administration, bulk amplicon-sequencing identified AAV6.2 and other AAV6 variants as well as AAV-DJ as the most efficient vectors for pulmonary gene expression in mice. Given the cellular heterogeneity of the lung, including several epithelial subtypes, mesenchymal cells and residential immune cells, for which AAV targeting efficiency has been largely unknown so far, we finally aimed to further develop the barcode sequencing approach towards single-cell resolution. Therefore, the barcoded AAV pool was applied i.t. into four C57BL/6 mice ($1.08x10^{12}$ vector genomes per animal), and a protocol for lung dissociation and high-viability single cell recovery was established. Following additional optimization of the Chromium 10x single-cell RNA-sequencing workflow by implementation of an additional cDNA library-based barcode amplification step, we were able to map the distribution of all AAV library variants to defined lung cell clusters and to identify the most efficient variants for each cell type. In summary, we established AAV transduction profiles for 34 AAV variants following i.v., i.p. and local lung delivery and further demonstrated feasibility of AAV biodistribution analysis at single cell resolution in the murine lung. Our data inform preclinical study design by enabling the selection of optimal vectors and administration routes for improved targeting of murine tissues and cell types of interest.

434 DNA-PKcs Inhibition Boosts rAAV Transduction in Polarized Human Airway Epithelia

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Recombinant Adeno-associated virus (rAAV) vectors have been widely used for therapeutic gene transfer in the studies of human gene therapy. rAAV vector drives persistent transgene expression in targeted tissues. AAV capsid directed evolution or rational design has evolved clinically desirable AAV capsids that exhibit higher transduction efficiency in tissues of cells, for example, AAV2.5T capsid was selected from the directed evolution of an AAV capsid library in human airway epithelia. In this study, we found rAAV2.5T transduction of well differentiated/polarized primary human airway epithelium (HAE) cultured at an air-liquid interface (HAE-ALI) induced a DNA damage response (DDR). The induced DDR was featured with phosphorylation of replication protein A32 (RPA32), histone variant H2AX (H2A histone family member X), and all three phosphatidylinositol 3-kinase-related kinases (PIKKs): ATM, ATR, and DNA-PKcs. While treatment of ATR pharmacological inhibitors or *ATR* gene knockdown inhibited rAAV2.5T transduction, DNA-PKcs inhibition or targeted gene knockdown increased rAAV2.5T transgene expression significantly in HAE-ALI. Notably, DNA-PKcs specific inhibitor NU7441 worked as a "booster" to further increase rAAV2.5T transgene expression after treatment of doxorubicin, whereas the epithelial barrier functions remained. Thus, our study provides evidence that DDR is associated with rAAV transduction in well differentiated HAE. Also, the study suggests that DNA-PKcs inhibition has the potential to boost rAAV transduction and that pharmacological compounds that block the DDR may be used as an augmenter to enhance rAAV transduction efficiency.

435 Primate-Conserved Carbonic Anhydrase IV and Murine-Restricted Ly6c1 Are New Targets for Crossing the Blood-Brain Barrier

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The blood-brain barrier (BBB) presents a major challenge to delivering large molecules to study and treat the central nervous system (CNS). This is due in part to the scarcity of effective targets for BBB crossing, the identification of which is the crucial first step of drug development. Here, we leveraged a panel of adeno-associated viruses (AAVs) previously identified through directed evolution for improved BBB transport to reverse engineer protein targets for enhanced BBB crossing. We identify both murine-restricted Ly6c1 and primate-conserved carbonic anhydrase IV (Car4; CA4) as novel receptors for crossing the BBB. We demonstrate how these receptors can unlock new experimental and computational target-focused engineering strategies by creating the enhanced Ly6c1-binding vector AAV-PHP.eC and by applying AlphaFold2-enabled in silico methods to rank capsids against identified receptors and generate capsid-receptor binding models. Here, with Car4, we add a completely new receptor to the very short list currently available for crossing the BBB in humans and, with Ly6c1, we validate a pipeline for receptor-targeted engineering. The identification of Car4/CA4 and structural insights from computational modeling provide new paths toward human brain-penetrant chemicals (drugs) and biologicals (including gene delivery).

436 The Potential Mechanism and Universality of the AAP Degron Model in the Process of AAV Capsid Assembly

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The formation of adeno-associated virus (AAV) capsid is a multi-step assembly process in which the AAV assembly activating protein (AAP) plays a crucial role at various steps including stabilization of AAV VP capsid monomers and subsequent transport of the VP capsid protein from the cytoplasm to the place in the nucleus where the virion formation takes place. Since persisted interaction between VP and AAP at the VP-VP interface would affect multimerization of VP proteins and hence hinder capsid formation, AAP must be removed effectively from VP proteins by a certain mechanism in the process of capsid assembly. In this regard, we have proposed the "AAP degron model" in which AAP's ability to promote self-disintegration once the VP-VP multimerization process is initiated, is critical for the assembly of VP proteins into capsids. To support this model, our lab has previously shown that the hydrophobic region (HR) in the N terminus of AAPs derived from AAV1, 2, 3, 5, 6, 7, 8, 9, 10 and 12 (i.e., AAP1, 2, 3, 5, 6, 7, 8, 9, 10 and 12) has a degron. However, it has remained puzzling that the HR in AAP4 and 11 does not show a degron activity, which does not align well with the AAP degron model. Here, we show that, despite the heterogeneity of the degron function in the AAP HR region among the different serotypes, the AAP degron model is still valid for AAV4 and 11; therefore, it is the universal model that explains effective removal of AAP in the capsid assembly process. This conclusion is supported by our new discovery that the conserved core (CC) region of AAP4 and 11 contains a degron. In addition, we could identify a single amino acid critical for the HR degron function. In the study, we compared amino acid sequences in the HR regions between degradation-prone HRs (AAP1, 2, 3, 5, 6, 7, 8, 9, 10 and 12) and degradation-resistant HRs (AAP 4 and 11) by sequence alignment and found that leucine (L) at position 25 (note: the amino acid position number is based on AAP9) is conserved across all degradation-prone AAP HRs except for AAP12 and the degradation-resistant HRs harbor threonine at the corresponding position. Using an AAP9HRL25T mutant fused to FLAG-GFP, we compared the GFP expression against the wild-type AAP9HR-FLAG-GFP. Unlike the wild type, the AAP9L25T mutant showed a retention of GFP expression albeit at a level lower than the FLAG-GFP control. Reciprocally, we found that GFP expression of AAP4HRT25L mutant showed a decrease in GFP expression compared to the wild-type AAP4HR-FLAG-GFP control. These observations demonstrate that the amino acid at position 25, which is either leucine or threonine, plays a critical role in the AAP's HR degron activity. Next, we investigated potential impacts of AAP retention on capsid assembly using the degradation-prone wild-type

AAP9 and the degradation-resistant AAP9L25T mutant. Consistent with the AAP degron model, the production of AAV9 VP3 particles was completely abrogated with the AAP9L25T mutant, indicating that persistent presence of AAP is detrimental to capsid assembly. Having obtained a plethora of evidence supporting the AAP degron model, our efforts were then directed towards identifying a degron in other AAP functional domains besides HR within AAP4 and 11 whose HR is devoid of a degron to reconcile our observations. Consequently, we confirmed that AAP4 and 11 indeed carry a degron, not in the HR, but in the CC region. We are currently working on identifying amino acid residues within the CC region of AAP4 and 11 responsible for degradation. These results significantly further our understanding of the function and mechanism of AAP in the process of capsid assembly.

437 A Viral Toolbox to Target and Manipulate Distinct Cortical Interneuron Subtypes

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Recombinant AAV (rAAV) can drive long-term gene expression *in vivo* and hence has become a popular gene delivery vector for research and gene therapy. However, to better leverage rAAV as a research and therapeutic tool, the expression of transgene needs to be regulated in a tissue/cell-type specific or context dependent manner. To address this, significant efforts have been made to modify the AAV capsid. However, the extend of specificity capsid choice can offer is relatively limited. Enhancers are non-coding elements that can regulate gene expression and can control the activity or spatial-temporal pattern of gene expression. In recent years, we and others have identified a number of enhancers that, when incorporated into rAAV vectors, can restrict the transgene expression to particular neuronal populations. Yet, viral tools to access and manipulate fine neuronal subtypes are still limited. Here, we performed systematic analysis of single cell ATAC-seq and RNA-seq data to identify enhancer candidates for each of the cortical interneuron subtypes. We then screened these candidates and successfully identified enhancers that are highly selective for distinct cortical interneuron populations in mouse and non-human primate. These enhancers, when used in the context of different effectors, can target (fluorescent proteins), observe activity (gCaMP), manipulate (opto- or chemo-genetics) and trace the connectivity (used in conjunction with mono-synaptic rabies) of specific neuronal subtypes. We also validated our viral tools across species. Thus, we present the development of a powerful set of tools for the study of cortical interneurons and the neural circuits to which they contribute. Moreover, our enhancer identification and validation strategies have the potential for use in tissue/cell-type specific or context dependent tools for therapeutic use.

438 Prenatal Administration of AAV9-GFP in Fetal Lambs Results in Widespread Biodistribution and Transduction of Female, but Not Male, Germ Cells

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Introduction: Prenatal somatic cell gene therapy could lead to treatment of early onset genetic disorders such as spinal muscular atrophy (SMA). Given the success of AAV9-SMN vector for the treatment of children with SMA, we tested the safety and biodistribution of AAV9-GFP in fetal lambs using a similar viral dose. **Methods:** Fetal lambs were injected with $1x10^{14}$ vector genomes/kg (equivalent to neonatal dose of AAV9-SMN) of scAAV9-GFP through the umbilical vein (UV, n=13) on e75 and harvested after 3 weeks or 2 months. 4 animals were injected intracranially (IC) through the cisterna magna. Vector biodistribution was evaluated using ddPCR for vector genomes (vg) and immunofluorescence. Toxicity analyses included maternal and fetal biochemistries and measurement of anti-AAV9 and anti-GFP antibodies. **Results:** Survival to harvest was >88% for both injected and uninjected; 3 fetuses (2 injected, 1 uninjected) in 2 litters suffered in-utero demise, likely secondary to chorioamnionitis. We detected widespread biodistribution of vg in all tissues examined (brain, spinal cord, liver, skeletal muscle, blood, placenta and gonads) with decreased levels at 2 months compared to 3 weeks ($p<0.05$ in heart, liver, diaphragm, lung, and lumbar spinal cord). Detection of vg in maternal ewes was low except in pregnancies with fetal demise. Widespread GFP expression in lamb tissues was observed at both time points including in multiple regions of the brain and spinal cord, diaphragm, and quadriceps muscles. We did not detect any histopathological toxicity in dorsal root ganglia. There was evidence of systemic toxicity including growth restriction, transient elevation of total bilirubin (in ewes and lambs), and histopathologic abnormalities in the liver, kidney, and cerebellum. We also detected anti-AAV9 (but not anti-GFP) antibodies in both ewes and lambs. Staining for the germ cell marker DDX4 demonstrated co-localization of GFP in ovarian germ cells (which also stained positive for the meiosis marker SYCP3); in contrast, GFP+ cells in testes did not stain with DDX4 or SYCP3. Integration analysis of gonads (3 female, 3 male) demonstrated integration of AAV in all samples, representing 40 different integration sites with very low (less than 7%) vector rearrangement rate. Integration events were not observed in genes previously associated with adverse events in gene therapy trials; however one of the samples showed integration in gene TMP3. **Discussion:** We confirmed widespread distribution of AAV9-GFP after systemic injection at a gestational age, comparable to the second trimester in humans. Robust GFP expression in spinal cord and muscle suggest potential benefits for patients with SMA or muscular dystrophy. However, prenatal administration of a dose that is equivalent to weight-adjusted clinical dose of AAV9-SMN resulted

in toxicity. Detection of vector in female, but not male germ cells could be secondary to the timing of double-strand DNA breaks associated with meiosis in fetal oocyte development, which could facilitate vector transduction and, possibly, integration. Lack of anti-GFP antibodies is encouraging for conditions in which antibodies against transgene-encoded proteins can limit efficacy. These results support the use of the sheep model for prenatal gene therapy experiments, including testing different vector doses and other strategies to limit toxicity; however, they also underscore the need to prevent germline integration.

439 Characterization of the AAV ITR Host Protein Interactome

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Recombinant Adeno-Associated Viruses (AAVs) are the leading choice for gene therapy delivery, with hundreds of clinical trials currently ongoing. However, hurdles remain before we can use this technology safely, with two main concerns being specificity and cellular toxicity. Cellular toxicity, mediated either by the immune response or by innate cellular defenses, is an ongoing struggle for the field. While these issues are complex, they stem partly from a common source: that the AAV interactome across different cell types is largely uncharacterized. In embryonic stem cells (ESCs) and neural progenitor cells (NPCs), AAV infection has been shown to activate DNA damage pathways, leading to apoptosis. Interestingly, in both cases, the induced apoptosis is dependent on the inverted terminal repeats (ITRs) of the transgene, which inspired efforts to engineer the ITR to prevent its interaction with host cellular factors. Here we set out to identify host factors that recognize the ITR, potentially enabling future engineering to prevent downstream cell signaling and improve transduction. We found that, when exposed to AAV, human NPCs have a distinct apoptotic phenotype while differentiated GABAergic neurons lose their apoptotic phenotype. To identify hNPC-unique ITR binders, we performed immunoprecipitation coupled with mass spectrometry (IP-MS) in lysates of hNPCs and hGABAergic neurons, stringently filtering the MS data to remove non-specific binding. We identified proteins involved in the DNA damage response and endosomal transport as well as components of the proteosome and spliceosome. Interestingly, many of these proteins were not selected in previous CRISPR screens for increased AAV transduction. By continuing to elucidate which host factors recognize the ITR and induce apoptosis in hNPCs, and extending our analysis to multiple cell types, we hope to establish the groundwork for an ITR interactome. This knowledge may facilitate the design of better transgenes to improve cell specificity, reduce cell toxicity, and make gene delivery safer and more efficient.

440 Generation of DNA Aptamers Against Pancreatic Surface Receptors Towards AAV Cell-Specific Targeting

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Adeno-associated virus (AAV) is a small DNA virus that has been closely studied for its potential for human gene therapy. There are multiple serotypes of AAV and recombinant AAV that have differential transduction efficiency and specificity towards different tissues, yet no cell type-specific AAV vectors have been created to date. In order to understand and improve cell/tissue transduction efficiency and specificity, our lab has been investigating the use of unnatural amino acid substitutions in combination with click-chemistry to conjugate AAV capsids with DNA and RNA aptamers that recognize specific cell surface receptors. Here, we utilized a version of Cell-SELEX (Systematic Evolution of Ligands by Exponential enrichment) in an attempt to generate aptamers that bind cell surface receptors. Specifically, we selected receptors reported to be present on pancreatic endocrine (GLP1R) versus exocrine (SCTR) cells. These receptors were exogenously expressed in a cell line to generate positive (+receptor) versus negative (native cell line) selection targets for Cell-SELEX. A random single-stranded DNA library underwent 10 rounds of selection, and at the conclusion of every selection cycle, a fraction of the library was taken for NGS analysis. Our preliminary analysis revealed progressively diminishing library complexity from ~$2x10^7$ to ~$9x10^5$ unique sequences from the initial to the final selection cycle, respectively. Upon filtering out rarer species represented below a 10^{-4} frequency, 803 unique sequences remained. Of these 803 sequences, many were similarly enriched when comparing between one receptor's selection versus the other receptor's selection, which likely represent sequences with non-specific binding to the cell line. For the remaining highly enriched sequences, additional steps are required to identify which sequences are the most promising candidates. Future steps include characterizing the affinity and specificity of these enriched sequences to the surface receptors of interest, and eventually using the selected aptamers for conjugation with AAV.

441 Targeting AAV Vectors to the CNS via De Novo Engineered Capsid-Receptor Interactions

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Viruses have evolved the ability to bind and enter cells through interactions with a wide variety of host cell macromolecules. We engineered peptide-modified adeno-associated virus (AAV) capsids that transduce the brain through the introduction of de novo interactions with two proteins expressed on the mouse blood-brain barrier (BBB), LY6A or LY6C1. The in vivo tropisms of these capsids are predictable as they are dependent on the cell- and strain-specific expression of their target protein. This approach generated hundreds of capsids with dramatically enhanced central nervous system (CNS) tropisms within a single round of screening in vitro and secondary validation in vivo thereby reducing the use of animals in comparison to conventional multi-round in vivo selections. The reproducible and quantitative data derived via this method enabled both saturation mutagenesis and machine learning-guided exploration of the capsid sequence space. Notably, during our validation process, we determined that nearly all published AAV capsids that were selected for their

ability to cross the BBB in mice leverage either the LY6A or LY6C1 protein, which are not present in primates. This work demonstrates that AAV capsids can be directly targeted to specific proteins to generate potent gene delivery vectors with known mechanisms of action and predictable tropisms.

442 High Throughput LCMS Characterization to Promote Chemically Engineered AAV Capsids Development

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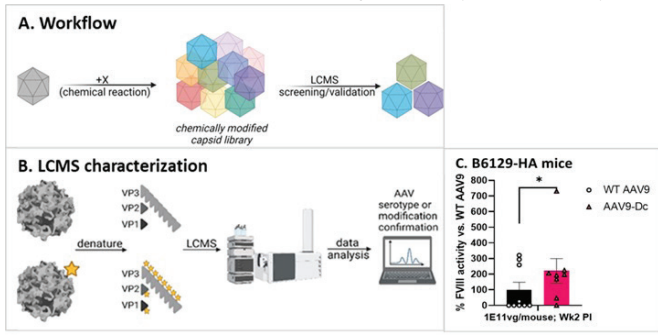
Adeno-associated virus (AAV) has emerged as a leading platform for gene therapy. With the skyrocketing rate of AAV research and the prevalence of many new engineered capsids being investigated in preclinical and clinical trials, capsid characterization plays a vital role in serotype confirmation and quality control. Further, peptide mapping the capsid proteins might inevitably be a future requirement by regulatory agencies since it is a critical step in good manufacturing practice (GMP) for biotherapeutic characterization. To overcome many challenges that traditional methods like SDS-PAGE and Western blots carry, liquid chromatography & mass spectrometry (LCMS) allows high resolution & sensitivity with great accuracy in characterizing the AAV capsid proteins. Our optimized LCMS method provides quick sample preparation, fast and high-throughput 4-min run, and high sensitivity, which allows for very efficient characterization of wild-type and engineered capsids. As a proof of concept, one top chemically engineered variant, AAV9-Dc carrying Factor-VIII, is shown to produce significantly higher gene expression in mice, compared to the unmodified AAV9 capsid. This study also reports the usage of LCMS/MS peptide mapping of AAV capsid proteins to determine the most accessible Lysine residues targeted by chemical modifications. Our protocols are anticipated to promote the development and discovery of novel AAV capsid variants with high accuracy and efficiency.



443 Strong Universal Micro-Promoters for Transgene Expression in Recombinant Adeno-Associated Viral Vectors

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Recombinant adeno-associated virus (rAAV) is a versatile and effective gene delivery tool for clinical gene therapy. However, the constrained packaging capacity of rAAV for cargo DNA up to 4.7 kb limits the diseases it can target. Here we report two unusually small promoters that enable the expression of larger cDNAs than standard promoters. These micro-promoters are only 84 bp (MP-84) and 135 bp (MP-135) in size but have strong activity in most cells and tissues. The sequence of the micro-promoters originates from the human insulin (MP-84) and glucagon promoter (MP-135). While the micro-promoters do not have an enhancer, AAV2 ITR is required for their promoter activity. We show here universal activity of MP-84 and MP-135 expressed from rAAVs in cultured cell lines, human pancreatic islets, and primary hepatocytes in culture, as well as in mouse brain and skeletal muscle *in vivo*. We further tested the capability of the micro-promoters to express DNA and RNA editing CRISPR-Cas systems in rAAV. The DNA editing rAAV expresses the spCas9 gene from MP-84 and the RNA editing rAAV the RfxCas13d gene from MP-135. These rAAVs also contain and package an expression unit for a guide RNA, since their final viral genome is smaller than 5 kb due to the small size of the micro-promoters. A proof-of-principle study targeting stably expressing EGFP showed efficient INDEL formations by the DNA editing rAAV. Hence, MP-84 and MP-135 will enable the therapeutic expression of genes and genetic engineering units that are currently too large for rAAV vectors.

444 Engineered Cardiac-Specific AAV Capsids from a Novel Next-Generation Platform (next-CAP) with Cross-Species Selection for Cardiac-Targeted Gene Therapy

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Background: Heart failure (HF) remains a leading cause of death and morbidity worldwide given the shortage of curative cardiac and cardiopulmonary transplantation. With HF incidence on the rise globally, targeting the underlying genetic and molecular foundation of the various HF types by viral-based gene delivery bears the potential to transform future clinical care of a relevant health care segment. In this regard, adeno-associated viruses (AAV) with engineered tropism for the heart are viewed as a key factor to generate solutions for safe and efficient delivery of therapeutic gene replacement,

silencing or editing systems to the diseased heart with minimized off-target organ transduction after a simple systemic administration **Technology & Results:** Here, we report the development of next-generation cardiac-specific AAV capsids using our experimental-bioinformatic AAV capsid eingineering platform (next-CAP) allowing for the serial high-throughput tracking and capturing of individual capsids across various tissues in different mammalian species (mouse, farm pig, non-human primate). A highly diverse capsid library with more than 100 million variants was generated from eight natural human AAV isolates integrating DNA shuffling and peptide display approaches. The library underwent a two-step *in vivo* evolution within the three animal models where in a first round cardiac-enriched capsid sequences were amplified from heart tissues serving as template to generate the AAV library for the second in vivo screening. By implementing a unique molecular identifier strategy for each capsid variant, we combined long- and short-term sequencing to achieve a comprehensive biodistribution resolution of all variants of interest. This allowed to capture novel AAV capsid variants displaying highest enrichment in hearts over all other organs, including the liver. In addition, we were also able to identify dual-organ AAV capsids showing preferred enrichment in a skeletal muscle/heart or brain/heart combinations. Subgroups of top heart-AAV capsid portfolio displayed an overall sequence homology of up to 99.5% identity further highlighting the presence of novel conserved heart-homing motifs. As producibility was another inherent selection criteria of our competitive in vivo screen, our cardiac-specific AAV capsid portfolio further provides scalable manufacturing characteristics. **Conclusion:** Our proprietary portfolio of novel heart-specific AAV capsids now enables the systematic development of optimal therapeutic capsid-promoter-transgene ensembles for further dose-expression, -efficacy and -toxicity assessment to the meet demands of the various hereditary and acquired HF indications in suitable model systems. Moreover, syndromes with secondary heart involvement (e.g., muscular dystrophy or neurological diseases) are amenable to our dual-organ capsids. In summary, we developed an efficient and versatile platform for the generation of next-generation AAV capsids for heart-specific gene therapy of yet uncurable rare and common cardiac diseases.

445 High-Throughput Generation and Identification of Novel AAV Capsids with Enhanced Properties for Gene Transfer Applications in the Central Nervous System

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Adeno-associated viral (AAV) vectors are effective vehicles in the delivery of genetic payloads for research and the treatment of diseases. There have been hundreds of naturally derived AAV capsids from tissues of humans, non-human primates (NHPs), and several other animals. AAV9 has emerged as the dominant vector in the development and translation of treatments targeting the central nervous system (CNS), but it has significant limitations including skewed biodistribution toward peripheral organs and the liver (as opposed to the CNS), and sub saturating CNS biodistribution even at the highest feasible doses. While many clinical trials continue to show success utilizing naturally occurring AAV capsids, the therapeutic window continues to be quite narrow with the limited tissue tropism they potentiate. Recombinant AAVs are exhaustively expensive and difficult to manufacture, and thus the manufacturing costs associated with high-dose human trials is one of the main prohibitive factors to initiate those studies. Aside from limitations in manufacturability, high doses of viral particles or unfavorable biodistribution can result in liver and dorsal root ganglion toxicity. These issues can be addressed with more efficient and better targeted capsid technology, which we aimed to address by generating novel AAV libraries combined with directed evolution. We show that coupling of directed evolution and single-molecule real time (SMRT) sequencing within mice and NHP establishes a pipeline to generate and identify novel capsids with superior characteristics compared to WT AAV9. We conducted a parallel screen in mice and NHPs utilizing mixed libraries of shuffled whole capsid chimeric AAVs, and AAV9-based libraries generated from targeted mutagenesis. Of note, the libraries involved modifications across the length of the capsid rather than localized peptide insertions, which was enabled by SMRT sequencing to gather high-content sequence data of the libraries and selected capsids. We utilized wild-type C57BL6, cell-type specific reporter mice, and NHPs with either intravenous or intrathecal lumbar puncture injection. Sequences of thousands of novel variants were recovered from both species across several CNS regions, cell types, and peripheral tissues. Hits were selected in an unbiased manner and top choices assessed for features such a manufacturability, in vivo tropism, and in vivo gene transfer efficiency. Our analysis thus far suggests a degree of species specificity among certain AAV variants, an observation that has plagued the discovery and translation of new viral vectors. However, we've also identified a number of capsid variants that appear to be positively selected for across both mice and NHPs. Our results suggest that libraries generated through whole capsid modification, coupled to advancements in sequencing technologies, may facilitate the generation and identification of novel AAV vectors in a high-throughput manner.

446 Novel Chemical Tyrosine Functionalization of Recombinant Adeno-Associated Viral Vectors to Improve Liver and Retina Gene Transfer Efficiency

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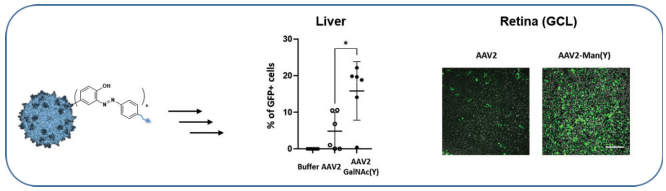
AAV gene therapy represents decades of biological and clinical research and still challenges need to be overcome to exploit all the potential of these vectors. Here, we focused on a chemical bioconjugation strategy to modify tyrosine (Y) residues on recombinant AAV capsids with carbohydrate ligands. The covalent coupling reaction was done by aromatic electrophilic substitution of the phenol of tyrosine residues with diazonium salt derivatives. *N*-acetylgalactosamine (GalNAc) sugar was used as an asialoglycoprotein receptor targeting unit for specific liver delivery, and the mannose sugar (Man) for targeting of specific retinal cells. After validation of the chemical coupling using a panel of analytical assays, we evaluated *in vivo*, in mice, the efficiency of rAAV2-GalNAc(Y) for liver and rAAV2-Man(Y) for retina in

comparison with rAAV2 (all vectors carrying an eGFP reporter gene expression cassette under the control of an ubiquitous promoter). In the liver, at one-month post intravenous injection, we observed significantly higher levels of GFP positive hepatocytes with a broader distribution with rAAV2-GalNAc(Y) than with rAAV2 (mean=16% and 5% of GFP positive cells, respectively). These data suggest a clear benefit of the tyrosine modified-rAAV2 capsid. In the retina, at one-month post subretinal-injection, the quantification of GFP fluorescence demonstrated a significantly higher number of fluorescent cells and overall density of the fluorescence after transduction with rAAV2-Man(Y) than with rAAV2. The ganglion cell layer (GCL) also showed increased fluorescence. This suggests either an improved uptake of the rAAV2-Man(Y) vector by ganglion cells, or a better diffusion of this vector through the tissue layers. Taken together, our findings reveal that the chemical remodeling of tyrosine on therapeutic vectors may become a valuable alternative to genetic engineering methods to improve organ tropism and protein expression in some specific cells. These results open up the use of the tyrosine modified vectors for gene therapy strategies targeting liver diseases, glaucoma and optic neuropathies.



447 Optimization of AAV-DARPin Fusions to Redirect Capsid Tropism

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Adeno-associated virus (AAV)-mediated gene therapy is a rapidly growing field with immense potential to deliver curative treatments to patients with unmet needs. For many indications, the utility of AAVs as delivery vectors is limited by their broad tissue tropism and lack of cell- and tissue-type specificity. This necessitates high doses to confer therapeutic benefit in the tissue of interest, thus increasing the risk of off-target effects and toxicity. In this study we developed chimeric AAV vectors that incorporate designed ankyrin repeat proteins (DARPins) engineered to target cell membrane receptors of interest. Athebody® DARPins are small "plug & play" binding proteins composed of ankyrin repeat units: an N- and C-terminal cap along with one to three internal repeats. Unlike full-length antibodies or antibody fragments, their small size (10-16 kDa), robust fold, high stability and lack of disulfide bonds make DARPins well-suited for incorporation into viral vector capsids. We optimized multiple parameters of the DARPin-AAV fusion construct and production including the VP insertion site, linker length, linker composition, VR mutations, and transfection conditions. DARPins of different repeat numbers, binding affinities, and receptor targets were also tested. We evaluated a set of receptors that can be harnessed to target the brain by ferrying molecules across the blood-brain barrier and other tissues. We chose one of these receptors as the target and selected high-affinity Athebody® DARPins by means of ribosome display, obtaining binders with a range of binding affinities down to picomolar affinity, and cross-reactivity with mouse, cynomolgus macaque, and human orthologs. Using our optimized AAV-DARPin construct, vector production titer and VP ratios could be maintained to near those of the unmodified vector. We found that DARPin insertions in VR-IV of VP1 could mediate >25-fold increased transduction of HEK293 cells over-expressing the target receptor compared to the unmodified vector. Insertions in VR-VIII of VP1 or at the N-terminus of VP2 increased transduction but to a lesser extent while the linker composition (glycine-serine linker vs proline-threonine linker) had minimal impact on transduction activity.

448 The Capsid Race: Multiplexed Analysis and Comparison of Rationally Engineered AAV CNS Capsids *In Vivo*

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Developing novel adeno-associated virus (AAV) capsids that can transduce cells in the central nervous system (CNS) via systemic administration is one of the most intriguing challenges in the field of AAV-based gene therapy. The inefficiency of blood-brain barrier (BBB) crossing and high-level sequestration and transduction of peripheral organs limit the CNS application of first generation AAV-based vectors due to the narrow therapeutic window. Therefore, several next generation AAV capsids have been engineered in recent years resulting in CNS capsid variants with a different mode of action. In this study, 26 wild-type serotypes as well as rationally designed CNS capsids were compared in a parallel and multiplexed 'race' *in vivo* to determine which capsid would transduce the brain most efficiently when administered systemically. The capsids were individually produced with each having a barcoded reporter transgene, pooled, and the resulting cocktail was intravenously (IV) administered to 7-week-old C57BL6 and BALBc mice. Two weeks after injection, both brain (frontal, caudal, cerebellum) and liver, as peripheral organ representative, were sampled. The barcoded reporter transgene was amplified by PCR and the resulting amplicons analyzed by next-generation sequencing (NGS) to assess the level of enrichment of each CNS capsid in each tissue. In addition, single-cell RNAseq (10X Genomics) was performed to determine the cell tropism of each capsid in each respective tissue of the CNS. Six of the engineered CNS capsids showed significantly enhanced transgene expression compared to wild-type AAV9 in both mice strains. In particular, CapX-38, a modified AAV9 capsid, showed between ~218 and ~33-fold higher brain transduction, depending on the mouse strain and brain region. Intriguingly, CapX-25, containing an additional modification in the VR-IV loop, presented itself with up to ~46-fold higher brain transduction, while showing ~37-fold decreased transgene expression

in liver of both mice strains. Single-cell sequencing results confirmed that transgene expression was mainly neuron-specific for both CapX-38 and CapX-25, showing their potential use for CNS gene therapy via systemic delivery. Utilizing this multiplexed 'race' analytical strategy can quickly identify the most promising capsid candidates for certain applications. Finally, the results of this study also highlight the importance of VR-IV loop in AAV capsid engineering to harness new capsids with interesting phenotypic features.

449 Biodistributional Study of Novel AAV-F Capsid vs AAV9 after Neonatal Intravenous Delivery

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AAV9 gene therapy has shown great pre-clinical & clinical efficacy, including Zolgensma for Spinal Muscular Atrophy. The use of systemic delivery of AAV9 gene therapy for neurological disorders requires the use of high vector titres to achieve a therapeutic effect. However, this can lead to off-target effects. Thus, the use of a different AAV capsid may be necessary to reduce off-target toxicity and ensure therapeutic efficacy in the central nervous system (CNS) and other systemic organs, with lower AAV volumes and concentrations. A novel synthetic AAV capsid, AAV-F has previously reported extensive CNS targeting after a single intravenous delivery to adult mice than its titre-matched paternal AAV9 capsid. However, this has not been replicated in neonatal mice. Our study aimed to investigate AAV-F capsid (containing a CAG promoter driving green fluorescent protein reporter (GFP)) after a single intravenous delivery to new-born mice, to establish biodistribution of vector for severe early-onset neurological disorders. Wild-type C57BL/6J mice were injected intravenously via superficial temporal vein within the first 24 hours of life with titre-matched AAV-F and AAV9 (1e12 vector genomes per ml). 5-weeks post-injection, treated mice and untreated controls were sacrificed and tissue were collected. Transduction efficacy was assessed by performing immunofluorescence. Using automated image analysis, the percentage of GFP-positive neurons and astrocytes were quantified. Our results showed AAV-F had a significantly higher expression profile of neuronal and astrocytic targeting throughout the brain when compared to titre-matched AAV9. In addition, there was greater targeting of visceral organs with AAV-F in comparison to AAV9. Overall, we have shown AAV-F to have a greater transduction efficiency within the CNS and systemic organs. Therefore, identifying AAV-F as an ideal capsid to treat early onset severe neurological disorders.

450 Efficient Transduction in Kidney Tubules with Minimum Off-Target Effects by Renal Vein Injection of Select AAV Capsids

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The advent of the next-generation sequencing (NGS)-based clinical diagnostics revealed unexpectedly high prevalence of monogenic kidney diseases not only in pediatric but also adult chronic kidney disease (CKD) cases, which has made CKD an attractive target for AAV vector-mediated in vivo gene therapy. However, gene delivery to the kidney has been challenging due to its low efficiency and inability to effectively target clinically relevant cell types such as renal tubular epithelial cells. Although recent progress in capsid engineering has led to generation of numerous AAV capsids with novel phenotypes, they have not yet been characterized in depth in the context of renal gene transfer. Especially, route of administration (ROA)-dependent differences in renal transduction profiles among different AAV capsids have yet to be investigated. Here we present a comprehensive profile of renal transduction of multiple AAV capsids delivered by two different ROAs, that is, intravenous injection (IV) and renal vein injection with the local blockage of the arterial, venous and urinary flow (RV), and show that effective transduction of renal tubules with minimizing off-target effects, which has not been possible by IV, could be attained by a combination of select AAV capsids and RV. In the study, we produced a DNA/RNA-barcoded AAV library containing a total of 47 AAV capsids, injected C57BL/6J mice with the library via IV or RV, and harvested the kidneys 6 weeks post-injection for the AAV Barcode-Seq analysis. This analysis revealed that AAV9 most efficiently transduces the kidney by IV among the 47 AAV capsids but AAV9 is outperformed by five AAV capsids including AAV-KP1 when administered by RV. Subsequently, we produced AAV9 and AAV-KP1 vectors expressing tdTomato under the control of the CAG promoter and injected them into mice via IV or RV to validate the Barcode-Seq observations and further characterize these capsids in renal gene transfer. The result showed that, although the renal transduction efficiency and cell type tropism of AAV9 remain the same regardless of ROAs, showing mesangial cell and interstitial cell transduction, AAV-KP1 can significantly enhance renal transduction by RV, showing >30 times better transduction than AAV9 only when RV is used. AAV-KP1 efficiently transduced cortical renal tubule epithelial cells, mostly proximal tubules by RV, while only mesangial cells were transduced by IV, emphasizing the importance of the choice of ROA and AAV capsid for effective transduction in renal tubules. In addition, by quantifying AAV vector genomes in the kidney and blood in mice shortly after AAV RV injection, we found that more vector genomes remained within the AAV-KP1-injected kidney than the AAV9-injected kidney, and accordingly, blood vector concentrations of AAV-KP1 vector were two orders of magnitude lower than those of AAV9, leading to much less vector spillover to non-target organs including the liver by RV injection of AAV-KP1. On the other hand, off-target transduction of AAV9 was not different between IV and RV. These results demonstrate that effective renal tubular transduction, which was not possible before, has now become attainable by careful selection of AAV capsid and ROA.

451 Our Proprietary Manufacturing Process, mAAVRx, Enables Improved AAV Manufacturing in Different Suspension HEK293 Systems

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Transient transfection (TT) of suspension HEK293 is one of the most robust AAV manufacturing platforms in the genetic medicines field due to its proven scalability and applicability to different therapeutic genes. The combination of novel plasmid design with optimized production process (within TT system) can further reduce manufacturing cost, which is one of the major challenges in the genetic medicines field. Previously, we reported that our proprietary manufacturing process, mAAVRx, achieves increased product yield, increased quality and more potent AAV vectors in scalable suspension HEK293 cells. For further improvement, we compared our novel plasmids to conventional three-plasmid system in two production systems: a) Expi293F cell line + transfection reagent FectoVIR; b) AAV-Max system with its own cell line, culture media, transfection reagent, enhancer and lysis buffer. Using the conventional three-plasmid system, FectoVIR in Expi293F cells outperformed the AAV-Max system. In contrast, using our proprietary manufacturing process, mAAVRx, the AAV-Max kit generated higher titers than Expi293F+FectoVIR. This trend was consistent across a series of different AAV capsids. In addition to the improvement in yield, further analysis of the quality of the purified vectors was also performed and compared. Using AAV-Max, mAAVRx led to a significant reduction in packaged plasmid DNA compared to the traditional three-plasmid system. The potency of the vectors was also assessed. These results help to further understand the quality of the vectors produced using mAAVRx. In summary, our proprietary manufacturing process, mAAVRx can be successfully applied to different production systems. The increase of the vector titers and vector purity could reduce the cost of goods and improve product quality, respectively.

452 Identification of AAV Variants Enriched in Skeletal Muscle and Muscle Stem Cells via Peptide-Display Library Screening

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Duchenne muscular dystrophy (DMD) is a monogenic disease caused by out-of-frame mutations in the *DMD* gene, which results in lack of dystrophin production. We have previously demonstrated efficient systemic delivery of CRISPR/Cas9 using adeno associated virus serotype 9 (AAV9) to achieve gene correction and dystrophin restoration in muscle tissues. However, the natural myonuclear turnover of skeletal muscle (SkMu) and inability of AAV vectors to efficiently target muscle stem cells (MuSCs), reduces the number of CRISPR-corrected myonuclei and dystrophin expression over time. We hypothesize that a lifelong therapy will require genomic correction of both SkMu and MuSCs, particularly when targeting the DMD pediatric population. Here, we develop and screen an AAV peptide-display library by modifying AAV serotype 9, by integrating a unique heptameric peptide on the capsid surface that corresponds to the packaged vector genome, in order to select for novel AAV capsid variants with enhanced tropism to SkMu and MuSCs. After two consecutive rounds of selection *in vivo*, using the *mdx* mouse model of DMD, we identified 42 MuSC-enriched and 37 SkMu-enriched capsid candidates with high fold enrichment scores after the first and second steps in the screening process. The 42 MuSC-enriched capsid variants contain a heptameric peptide consensus sequence overrepresented by glycine amino acids while the 37 SkMu-enriched heptameric candidates are dominated by glycine and serine amino acids. In addition, we determined 8 of the 42 MuSC-enriched and 5 of the 37 SkMu-enriched capsid candidates contained low fold enrichment in off-target organs, such as the liver, which may suggest a capacity for liver de-targeting. Lastly, our analysis confirmed 9 shared capsid variants that are highly enriched in SkMu and MuSCs, which are being prioritized in ongoing validation studies to determine which AAV capsid variants have superior transduction efficiency to SkMu and MuSCs compared to the parental AAV9 as well as novel AAV variants, such as AAVMYO, recently identified to be SkMu-tropic. Identifying skeletal muscle and muscle stem cell-tropic AAVs offers the potential to provide a long-lasting supply of CRISPR-corrected muscle stem cells and a life-long sustainable therapy for DMD and other neuromuscular disorders.

453 AAV Transduction of MSCs is Enhanced by Genetic Engineering Medium

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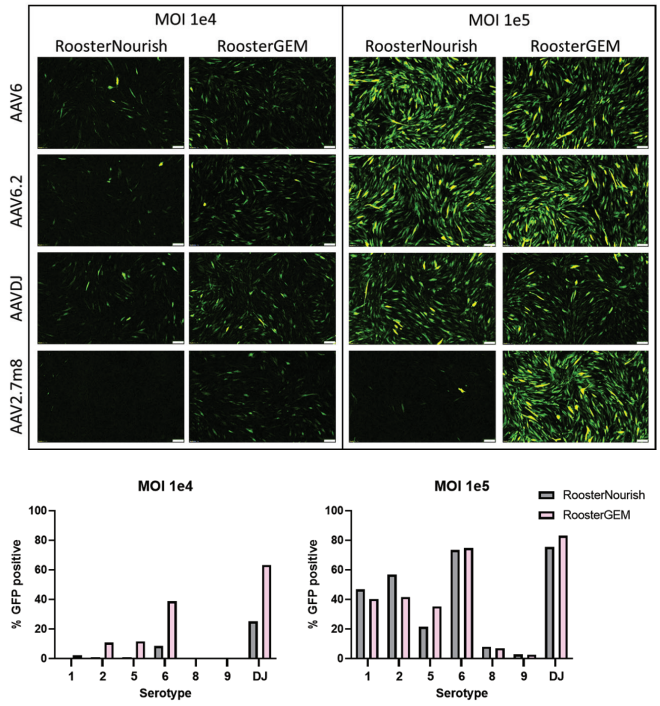
RoosterBio, Frederick, MD

Introduction Adeno-associated virus (AAV) is currently being used in clinical trials for a variety of indications due to its safety and efficacy. AAV gene transfer to Mesenchymal stromal Cells (MSCs) has been shown to enhance a wide range of MSC functions *in vivo*. For example, *in vivo* administration of AAV-modified MSCs has been shown to increase neuroprotective effects, promote bone regeneration, and decrease immune rejection. Despite the prevalence and promise of AAV, existing literature shows variability in AAV transduction efficiency and optimal serotype for MSCs. We have previously demonstrated that a genetic engineering medium increases the transduction/transfection efficiency of MSCs with lentivirus/mRNA. Our goal herein was to determine which AAV serotype(s) transduce(s) MSCs and to explore if a genetic engineering medium can enhance AAV transduction of MSCs. **Methods** Bone marrow-derived MSCs (BM-MSCs) were plated such that they were 80% confluent the following day. AAV from one of two vendors was diluted in RoosterNourish growth medium or RoosterGEM genetic engineering medium to the appropriate MOI, then added to cells. After an overnight incubation, medium was exchanged to fresh growth medium. Transduction efficiency was evaluated using widefield microscopy and flow cytometry. **Results & Discussion** In an initial screening, AAV serotypes 1, 2, 3, 4, 5, 6, 6.2, 7, 8, 9, rh10, DJ, DJ/8, PHP.eB, PHP.S, 2-retro, QuadYF, and 2.7m8 were evaluated at MOI 1e4 and 1e5. At MOI 1e4, AAV6, AAV6.2, and AAVDJ were observed to transduce MSCs in growth medium, but genetic

engineering medium enhanced transduction efficiency (Figure 1). Additionally, genetic engineering medium enabled the transduction of serotype AAV2.7m8. At MOI 1e5, AAV6, AAV6.2, AAVDJ, AAVPHP.S, and AAVquadYF were observed to transduce MSCs in growth medium and genetic engineering medium (Figure 1). genetic engineering medium also enabled the transduction of MSCs with serotypes AAV2, AAV3, and AAV2.7m8. We then transduced MSCs using AAV sourced from a different vendor to ensure consistency between AAV preparations. In this case, AAV serotypes 1, 2, 5, 6, 8, 9, and DJ were evaluated at MOI 1e4 and 1e5. At the lower MOI of 1e4, AAV2, AAV5, AAV6 and AAVDJ transduction were enhanced by genetic engineering medium, compared to growth medium (Figure 2). At the higher MOI of 1e5, all serotypes transduced MSCs with roughly the same efficiency in genetic engineering medium as in growth medium. AAV8 and AAV9 only sparsely transduced MSCs. In summary, AAV6 and AAVDJ are the optimal serotypes for the BM-MSCs herein. Additionally, genetic engineering medium enhances AAV transduction at low MOIs, but does not provide added benefit once the MOI reaches a certain high level. Future work aims to transduce MSCs with a functional gene to modulate MSC phenotype.



454 Development of Genome-Modified Generation Z (GenZ) Single-Stranded AAV Vectors with Improved Transgene Expression in Human Cells *In Vitro* and in Mouse Liver *In Vivo*

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The wild-type (WT) AAV contains a single-stranded DNA genome, and expresses its genes poorly, because there is no host cell RNA polymerase that can transcribe a single-stranded DNA. Similarly, transgene expression from recombinant ssAAV vectors is also largely sub-optimal since the viral second-strand DNA synthesis is strongly inhibited by phosphorylated forms cell host chaperone protein, FKBP52, binding to the D-sequence at the 3'-end (*Proc. Natl. Acad. Sci., USA*, 94: 10879-10884, 1997). It has not been possible to delete the D-sequence at the 3'-ITR as it serves as the "packaging signal" for the AAV genome (*J. Virol.*, 70: 1668-1677, 1996). Previously, we also reported that the distal 10-nucleotides (nts) in the D-sequence are dispensable (*J. Virol.*, 71: 3077-3082, 1997). In the current studies, we generated an N10-sequence library in which the proximal 10-nts in the D-sequence were replaced with random 10-nts to test the hypothesis that one or more of these N10-sequences will not only allow successful packaging of the AAV genome, but also evade FKBP52-mediated inhibition of the viral second-strand DNA synthesis, thereby allowing robust transgene expression from ssAAV vectors. One such sequence, 5'-ATGTGCTTGA-3', was identified that allowed successful rescue, replication, and packaging of the AAV genome. This sequence was inserted in a recombinant AAV2 genome replacing the proximal 10-nts in D-sequence at both ITRs flanking an expression cassette containing a firefly luciferase-enhanced yellow fluorescent protein (FLuc-EYFP) under the control of the chicken β-actin (CBA) promoter, designated as generation Z ("GenZ") ssAAV vector. Transduction efficiencies of wild-type (WT) and GenZ ssAAV2-CBAp-FLuc-EYFP vectors were evaluated in human HeLa cells *in vitro*, the results of which are shown in **Figure 1A**. The extent of the transgene expression from the GenZ AAVrh74 vectors was ~20-fold higher than that from the WT AAVrh74 vectors. Transduction efficiencies of WT and GenZ ssAAV2-CBAp-FLuc-EYFP vectors were also evaluated in C57BL6/J mice following intravenous delivery *in vivo*. As can be seen in **Figure 1B**, the GenZ ssAAVrh74 vectors averaged ~5-fold increase in transgene expression in the liver, compared with that from the WT ssAAVrh74 vectors. The observed increase in transgene expression was not as pronounced *in vivo* since AAVrh74 vectors are closely related to AAV8, and ssAAV8 vectors transduce mouse liver very efficiently. Taken together, these studies document that (i) A GenZ ssAAV DNA genome has been created that overcomes the problem of viral second-strand DNA synthesis; and (ii) The GenZ ssAAV vectors behave more like scAAV vectors, but without the size-limitation. The GenZ ssAAV DNA genome can be packaged into any AAV serotype capsid vector; and packaging of the GenZ ssAAV DNA genomes in capsid-modified NextGen AAV serotype vectors should further enhance the performance of Opt^{Z} vectors, the availability of which has significant implications for their potential use in achieving high-efficiency transgene expression of larger genes.

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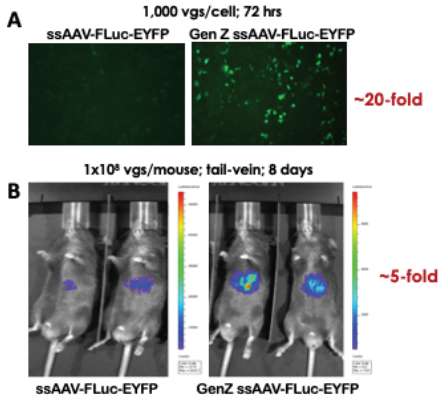

Figure 1: Transduction efficiency of WT and GenZ ssAAVrh74 vectors in human HeLa cells *in vitro* **(A)**, and in C57BL/6 mice *in vivo* **(B)**. HeLa Cells were transduced with each vector at 1,000 vgs/cell and transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. Approximately $1x10^8$ vgs of each vector were injected via the tail vein and whole-body bioluminescence images were obtained 8-days post-vector administration.

455 *In Vivo* Selection in Non-Human Primates Identifies AAV Capsids Having Enhanced Transgene Expression and Biodistribution in Spinal Cord after Intrathecal Administration

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Systemic administration of adeno-associated virus (AAV) vectors for spinal cord gene therapy has challenges including toxicity at high doses and pre-existing immunity that reduces efficacy. Intrathecal delivery of AAV vectors into the spinal cord can avoid many of the issues of systemic delivery, although achieving broad distribution of the vector and transgene expression throughout the spinal cord is challenging and leakage to the periphery may occur. Here we performed two rounds of *in vivo* biopanning in non-human primates with an AAV9 peptide display library injected intrathecally and performed insert sequencing on DNA isolated from either whole tissue (conventional selection), isolated nuclei, or nuclei from transgene-expressing cells. A subsequent barcoded pool of 13 candidates and AAV9 was compared at the DNA (biodistribution) and RNA (expression) level in spinal cord and liver. Most of the candidates displayed enhanced biodistribution compared to AAV9 at all levels of spinal cord ranging from 2 to 265-fold. Nuclear isolation or expression-based selection yielded 4 of 7 candidate capsids with enhanced transgene expression in spinal cord (up to 2.4-fold), while no capsid obtained by conventional selection achieved that level (0 out of 6). Furthermore, several capsids displayed lower leakage to the liver of up to 1,250-fold, compared to AAV9, providing a remarkable on target/off target biodistribution ratio. Currently, we are evaluating these capsids' transduction performance (transduction efficiency, cellular tropism) after intrathecal injection in mice. The selection method described here should be useful in clinically relevant large animal models.

456 Successful Production of Monoclonal Antibodies Against Fully Assembled AAV Anc80 Capsid by a Simple DNA Vaccination Method

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With the advent of technologies that can effectively create genetically engineered AAV capsids with novel biological phenotypes, the repertoire of novel AAV capsids has significantly expanded. Monoclonal antibodies (mAbs) against these novel AAV capsids would provide useful reagents to study them; however, conventional methods for the production of mouse mAbs are often expensive and complex. Accordingly, the resource of mAbs for newly emerged genetically engineered capsids still remains very limited. Here, we report a novel, simple and inexpensive method for effectively producing mouse mAbs against fully assembled AAV capsids by intravenous injection of plasmid DNA into mice and demonstrate proof of concept of the method by successfully generating anti-AAV Anc80 mouse mAbs that react specifically with fully assembled AAV Anc80 capsid, which do not currently exist. In this method, DNA vaccination is accomplished by producing fully assembled AAV capsids in hepatocytes in mouse liver by hydrodynamically delivering the following two plasmid DNAs via the tail vein: one expressing AAV VP3 protein and the other expressing AAP under the control of the CMV promoter. In the study, to immunize mice with AAVAnc80 viral capsids, 10 eight-week-old BALB/c female mice were injected with 50 microgram each of pCMV-AAVAnc80VP3 and pCMV-AAP2 plasmids in 100 mL/kg saline via the tail vein using a hydrodynamic injection technique at days 0, 14, 21, 28, and 35. At the 3-week time point, serum samples were collected and anti-AAVAnc80 antibody titers were assessed by an AAVAnc80 capsid antibody ELISA and an HEK293-based neutralizing antibody (NAb) assay using an AAVAnc80-CMV-luc vector. The result demonstrated that the animals developed antibodies against AAVAnc80 capsids and all of them showed neutralizing activities. At the 6-week time point, 2 mice that showed high titers of NAbs against AAVAnc80 were selected for the generation of monoclonal antibody-producing hybridoma cell lines. The spleens were harvested from these two mice and splenocytes were then isolated and fused with a mouse myeloma cell line. By screening approximately 700 hybridoma clones by ELISA, we obtained a total of 20 candidate clones producing mAbs. Among them, 9 mAbs were fully characterized for their ability to bind fully assembled capsid (f+ or f-), their ability to bind monomer (m+/m-), their ability to neutralize (n+/n-), cross-reactivity against AAV2, 5 and 9 (c+/c-) and isotypes. Please note that f+ and f- indicate the presence and absence of the ability to bind fully assembled capsid, respectively, and the other designations (m, n and c) follow the same. As a result, we could obtain mAbs that can be categorized into the following four distinct groups: (1) f+m-n+c-, (2) f+m-n+c+, (3) f+m+n-c-, and (4) f+m+n-c+. Using the 1B11 and 2A7 mAbs (Category 1 mAbs) that

can specifically recognize fully assembled AAVAnc80 capsids and do not cross-react with other AAV serotypes, we have successfully established fully assembled capsid-specific AAV Anc80 ELISA that does not cross-react with other serotypes. This method can be readily applicable to production of mAbs against any AAV serotypes and emerging genetically engineered AAV capsids for which mAb reagents are not currently available.

457 Protein Expression/Secretion Boost by a Novel Unique 21-mer c*is*-Regulatory Motif (Exin21) via mRNA Stabilization

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Boosting protein production is invaluable in both industrial and academic applications. We discovered a novel expression-increasing 21-mer *cis*-regulatory motif (Exin21) that inserts between SARS-CoV-2 envelope (E) protein-encoding sequence and luciferase reporter gene. This unique Exin21 (CAACCGCGGTTCGCGGCCGCT), encoding a heptapeptide (QPRFAAA, designated as Qα), significantly (34-fold on average) boosted E production. Both synonymous and nonsynonymous mutations within Exin21 diminished its boosting capability, indicating the exclusive composition and order of 21 nucleotides. Further investigations demonstrated that Exin21/Qα addition could boost the production of multiple SARS-CoV-2 structural proteins (S, M, and N) and accessory proteins (NSP2, NSP16, and ORF3), and host cellular gene products such as IL-2, IFNγ, ACE2, and NIBP. Exin21/Qα enhanced the packaging yield of S-containing pseudoviruses and standard lentivirus. Exin21/Qα addition on the heavy and light chains of human anti-SARS-CoV monoclonal antibody robustly increased antibody production. The extent of such boosting varied with protein types, cellular density/function, transfection efficiency, reporter dosage, secretion signaling and 2A-mediated auto-cleaving efficiency. Mechanistically, Exin21/Qα increased mRNA synthesis/stability, and facilitated protein expression and secretion. These findings indicate that Exin21/Qα has the potential to be used as a universal booster for protein production, which is of importance for biomedicine research and development of bioproducts, drugs and vaccines.

458 Characterization of Adeno-Associated Virus Composed of N-terminal Clipped VP3

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rAAV capsid is composed of three viral proteins (VPs): VP1, VP2, and VP3. The VP composition of rAAV capsid is estimated to be a molar ratio of 1:1:10. To ensure the safety and efficacy of rAAV drugs for human gene therapy, the stoichiometry of VPs of rAAV vector should be carefully monitored and controlled. Recently, highly sensitive analytical techniques have identified that Clipped VP3, which is 8 amino acid residues shorter than VP3, is present in low amounts in addition to the conventional VPs in many serotypes. Clipped VP3 is suggested to be generated by leaky scanning, in which the ribosome misses the VP3 start codon, however the effect of Clipped VP3 generation on the VP assembly remains unclear.In this study, we investigated the strategy to promote, or suppress Clipped VP3 generation in rAAV vector. Characterization of the AAV variants was performed, including stoichiometry of VPs were determined by capillary gel electrophoresis (CGE), amino acid analysis of VPs was measured by LC-MS, transduction efficacy assay using HelaRC32 and HEK293 cells.

459 An Integrated Adeno-Associated Vector Development Platform for Inner Ear Disorders

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Background: The inner ear is a diverse mosaic of highly specialized cells including sensory hair cells, supporting cells and spiral ganglion neurons, all of which play an important role in the process of hearing. More than half of the congenital non-syndromic deafness cases have a genetic cause that affect specific cell populations in the cochlea. Gene therapy is a promising avenue for those patients, but requires precise spatiotemporal transgene expression. AAV-based gene transfer shows great potential for the treatment of hearing loss, as they provide stable gene expression over long period of time in post-mitotic cells and are relatively safe to use. Furthermore, AAV cell-specific tropism and transgene expression patterns can be fine-tuned through AAV capsid engineering and the addition of regulatory sequences in the expression cassette. Development of new AAV gene therapy programs for hearing loss is dependent on the efficient screening of new capsids and vectors of interest targeting specific cell populations within the cochlea, and on the subsequent production of the candidates to be evaluated. **Method:** We developed an in-house research grade AAV production and capsid purification protocol in small scale cultures, eliminating the need for specialized equipment such as bioreactors and ultracentrifuge, and greatly speeding up productions while cutting down on costs. This protocol yields high viral titers comparable with commercial AAV productions (range: 5E+12 to 5E+13 VG/mL, depending on the AAV serotype), show very minimal endotoxin contamination, high AAV purity, and is therefore compatible with *in vivo* use. We observed high transduction efficiency and low toxicity *in vitro* on HEK293T cells and ex vivo on murine organ of Corti explants. Injections of these AAV in the inner ear through the round window membrane of p15 mice is well tolerated and leads to widespread transduction of multiple cell types within the organ of Corti, stria vascularis and the vestibule. As expected, the transduction profile is dependent on the AAV serotype. In order to expand our molecular toolbox to precisely target cells of interest in the cochlea, we generated a library of new synthetic capsids through domain shuffling and used those vectors to encapsulate an eGFP expression cassette under the control of a cytomegalovirus (CMV) ubiquitous promoter to track cell transduction. Using our integrated AAV screening platform, we are

able to rapidly produce the new AAV "in house", inject them in the cochlea of p15 mice and assess the transduction profile 2 weeks later. Promising AAV candidate showing transduction of cell types of interest are further modified to improve specificity. Tropism and transduction efficacy studies are completed by local tolerability studies, audiometry (including auditory brainstem responses (ABRs), distortion product otoacoustic emissions (DPOAEs) recordings). Behavioral startle response tes[LD1] ts are also performed to ensure that sound signal integration is not disrupted by the surgery and/or the viruses. **Conclusion:** In summary, we have developed an integrated AAV discovery platform that allow us to design new AAV vectors tailored for addressing a variety of inner ear pathologies and to test for their specificity and efficacy *in vivo*

460 Diversity Analyses in AAV Libraries Containing Peptide Insertion Using Short- and Long-Read Next-Generation Sequencing Approaches

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Specificity and efficiency are key factors in the design of viral vectors in gene therapy. In the field of adeno-associated viruses (AAV) vectors, several approaches have been investigated to create variants that target different tissues with high specificity and efficiency. Insertion of peptide ligands into variable regions (VR) of the AAV capsid, enabled vector de-targeting from off-target tissues as well as improvement of on-target cell transduction. Early development of these modified vectors includes the initial design and production of an AAV library containing up to millions of variants with differently recombined peptide insertions in the VRs. Quality control is essential to confirm the intended diversity of the library and to also rule out any biases for certain variants before going to in-vivo experiments. Here we investigate a short-read (Illumina) and a long-read (Oxford Nanopore) 2nd and 3rd generation sequencing approach for diversity estimation. We used our previously designed plasmid library containing 6000 different peptides inserted in both VR-IV and VR-VIII leading to a theoretical diversity of 3.6E+07 variants. We generated amplicons covering both peptide insertions from both the plasmid and AAV libraries. After library preparation, both Illumina and Nanopore sequencing will be performed, followed by subsequent bioinformatic analysis. Here, the generated reads of both approaches will be aligned to the reference containing all possible variant sequences. This approach allows us to estimate the diversity of the library in both the plasmid and the virus configuration. Potential biases towards over-representation of single variants will be detected before application to in-vivo experiments. The same approaches will be performed after injection of an AAV library into animals to monitor the specificity of the variants by analyzing different tissue samples. In summary, we show a comparison of short- and long-read NGS approaches to estimate AAV library diversity and an approach for tracking potential biases of peptide insertion variants that may lead to improved specificity and efficiency of AAV vectors.

461 *In Vivo* Gene Delivery Using Exosomes Engineered to Contain AAV Improves Transduction Efficiency and Resists Neutralizing Antibodies

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Background: Adeno-associated virus (AAV) is a commonly used gene therapy vector due to its excellent safety profile, stability, and duration of transgene expression. One major limitation of current AAV vectors are neutralizing antibodies (nAb), which are present in many potential patients (preventing treatment) and develop in patients following an initial dose (preventing re-administration). Packaging within exosomes (exoAAV) is a potential strategy to protect AAV from antibody-mediated neutralization, allowing efficient transduction even in the presence of high nAb titers. We have developed a method for engineering exosomes to package AAV (engEx-AAV), resulting in a potent vector that resists antibody-mediated neutralization and shows strong transduction capacity when administered in vivo. **Methods:** A camelid nanobody (VHH) phage display library was constructed from PBMCs isolated from an alpaca immunized with AAV9. The library was panned for pH-sensitive binders and twelve sequences were identified. VHH sequences were produced as recombinant proteins and confirmed to bind AAV9 using an ELISA assay. Stable cell lines were generated expressing a VHH sequence fused to an EV-localizing peptide derived from BASP1. Cells were transiently transfected with plasmids for AAV production and cultured for three days before engEx-AAV purification by differential and density gradient ultracentrifugation. AAV loading into exosomes was evaluated by qPCR, Western Blot, and ELISA. In vitro transduction assays were performed using HeLa cells to assess transgene expression. To determine the relative activity of different preparations in vivo, AAV9 or engEx-AAV9 were administered intravitreally into the eyes of rodents and transgene expression assessed using immunohistochemistry. **Results:** Exosomes purified from cells stably expressing BASP1-VHH fusions demonstrated roughly equivalent protein levels of all VHH constructs, as assessed by Western blot. However, despite containing similar levels of VHH protein, considerable differences were observed in the amount of AAV present in purified exosomes, ranging from equivalent to un-engineered exoAAV up to ~100-fold higher, as assessed by qPCR. Western blot analysis showed that the level of AAV capsid protein correlates with the amount of AAV transgene DNA across samples. In vitro transduction assays showed variable responses, with many of the VHH sequences failing to efficiently transduce cells while others exhibited robust transgene expression at levels significantly above that observed with either AAV or non-engineered exoAAV. Upon Intravitreal administration, engEx-AAV demonstrated improved transduction across all layers of the retina compared to dose-matched AAV. **Conclusions:** Engineering exosomes to carry an AAV-binding ligand can dramatically increase the amount of loaded AAV. We identified three VHH sequences that were able to load AAV into exosomes and allow efficient transduction of recipient cells. Our results

show that production of functional, nAb-resistant exoAAV can be greatly increased by optimized engineered exosomes. In vivo studies demonstrated improved and uniform transduction across all layers in the retina when AAV was packaged inside exosomes.

462 Application of Cas-9 Guided Adapter Ligation (nCATS) and Long Read Sequencing to Assess Quality of Gene Therapy Products

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Gene therapy products use polynucleotide constructs, containing the transgene, which is packaged into a protein-based nanoparticle to deliver the transgene to cells. Polynucleotide transgenes used for gene therapy can be constructed using flanked inverted terminal repeats (ITRs) around the transgene of interest. The ITRs help form concatamers that can persist as episomes in transduced cells. A gene therapy product; however, may contain undesirable constructs that have fragmentation, changes in cassette orientation, damaged or truncated elements, translocations or other abnormalities. These changes may result from the transfection process or the actual transgene construct. These impurities may render the transgene ineffective, contribute to genotoxicity, the need for higher doses and production of larger production quantities because the effective dose with an intact transgene is reduced. We found polynucleotide transgenes containing ITRs can provide regions of duplexed DNA which can serve as adapters. We then applied targeted Cas-9 guided ligation adapters (nCATS) from which we performed long read Nanopore sequencing. Using a model 4.8 kilobase (kb) AAV vector, we identified batches were the actual polynucleotide constructs varied in size from less than 500 bases up to 4.42 kb. We quantified the number of fragments along this distribution to show how much of the intended transgene is actually in the product. Our method simultaneously assessed single nucleotide variants (SNVs), structural variants and CpG methylation in a single reaction using only 3 ug or less of genomic DNA. Sequencing results showed orientation of the transgene elements, nucleotide and structural variants to show what the actual product contains. We feel this method can be used to assess the purity, provide quality assurance and quality control for gene therapy agents used in human gene therapy trials and quality control between different batches and transgene experiments.

463 Engineering Novel AAV Capsids for Cardiac Gene Delivery

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Gene therapy is an emerging treatment option for both acquired and inherited cardiac disorders. While certain known adeno-associated virus (AAV) serotypes can achieve moderate transduction of the heart, the requirement of high doses and the substantial viral load to the liver or off-target cell types raise the critical need for novel AAV capsids with improved properties. We have established in-house AAV capsid engineering capabilities and successfully screened more than 30 diverse, proprietary AAV libraries representing more than one billion unique capsids in multiple *in vitro*, *in vivo*, and *in silico* models to discover novel AAV capsids that can target the different cardiac cell types through different routes of administration. Targeting cardiomyocytes following systemic delivery is critical to enable gene therapy treatments for many cardiac conditions and AAV9 is currently the workhorse capsid for this type of gene therapy. To identify novel capsids that outperform AAV9, we performed two rounds of directed evolution studies in NHPs with AAV libraries built on multiple parental capsids focusing on selecting novel capsids that efficiently transduce the heart while being de-targeted from the liver. We further compared 102 candidates using pooled, RNA/DNA-barcode-based methodology in NHPs, mice, pigs, and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and identified multiple novel AAV capsids with superior properties including improved heart-to-liver ratio, improved cardiomyocyte transduction, and consistency between different species. Finally, we scaled up the production of our top novel capsid candidates with a clinically relevant manufacturing platform and validated their improved heart-to-liver tropism in NHPs at a clinically relevant dose. Together, these novel AAV capsids may enable more efficacious and safer next generation gene therapies for cardiac disorders.

464 Stepwise Evolution of the AAV5-Derived Capsid VCAP-100 Identifies Novel Variants with Improved CNS Transduction and Liver Detargeting Following Systemic Injection

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Adeno-Associated Virus (AAV)-gene therapy shows great promise to treat a wide array of human genetic diseases. However, current therapies are limited by target tissue transduction and liver-associated toxicity, especially when administered systemically. Recent efforts aiming at engineered AAV capsids for improved transduction of the central nervous system (CNS) have been largely focused on AAV9, partly because of the propensity of this serotype to cross the blood-brain barrier more easily than other AAVs. Alternative AAV serotypes, however, could offer unique features in terms of tropism, tissue specificity, manufacturability or immunogenicity. We focused our engineering efforts on AAV5, a highly divergent AAV serotype with a low prevalence of preexisting neutralizing antibodies in the general population. We previously reported the discovery of VCAP-100, an AAV5 derived capsid with improved CNS transduction from the intravenous (IV) route in both non-human primate (NHP) & rodent species. In this study, we aimed at 1) further improving VCAP-100's CNS transduction, and 2) reducing liver transduction to improve AAV safety profile. Leveraging our proprietary RNA-driven TRACER

evolution platform, we further evolved various surface regions of VCAP-100 and AAV5 capsids that may play a role in brain and liver tropism. To increase the probability of maintaining the cross-species tropism properties of VCAP-100, biopanning was performed in parallel in NHPs and rodents. We identified several families of capsids based on sequence homology, improved brain tropism, and liver detargeting. In our pooled capsid library assays, one particular family of VCAP-100 derivatives displayed up to 300-fold liver detargeting and 6-fold improved brain transduction in NHP over the parental sequence. Another family of AAV5 derivatives included variants with up to 1700-fold improved brain transduction in NHP and 45-fold in rodent over the AAV5 serotype. These novel capsids will be further validated individually to determine cellular tropism. Interestingly, most variants only exhibited a limited number of amino acid substitutions, supporting the idea that significant tropism improvement can be achieved with minor changes in capsid surface residues. Overall, this study identified new VCAP-100 liver detargeted variants and CNS tropic AAV5 derivatives that further validate our proprietary TRACER platform, and could display an improved safety profile.

465 Rapid Quantification of Capsid Content of Adeno-Associated Viruses (AAVs) by Capillary Electrophoresis-Based Western Analysis

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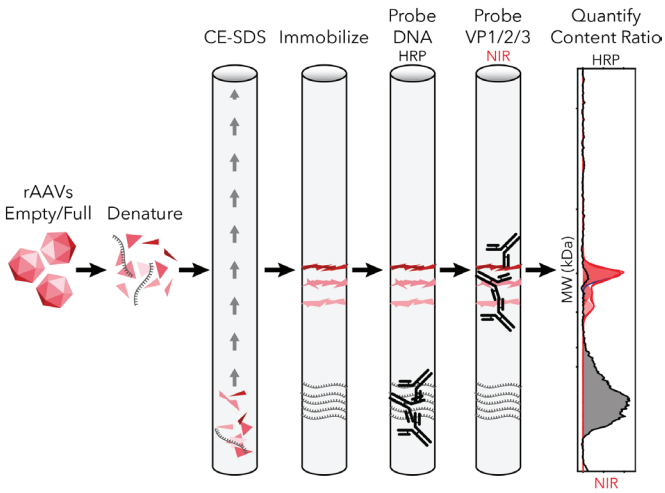
Gene therapy utilizing Adeno-associated viruses (AAVs) is on the rise, with two AAV-based treatments already approved by the FDA and many more in clinical development. AAVs are a potent gene delivery tool, but their complexity lies in their two key components: the proteinaceous capsid (composed of VP1, VP2, and VP3) and the contained DNA cargo. During AAV manufacturing, monitoring the number of fully loaded particles versus empty or partially filled ones is crucial as it affects their efficacy. Currently, determining the empty/full status of an AAV sample is a challenge, and most methods require significant amounts of purified sample. To address this, we introduce a new approach for measuring both the DNA and capsid protein content of AAV particles using a capillary-based western blotting platform (Simple Western). This method separates VP1/VP2/VP3, and the capsid DNA based on size and quantifies them using immunodetection. Our results demonstrate strong correlation with the % full obtained by orthogonal techniques. With good sensitivity, linearity, and the ability to analyze 24 samples in just 5.5 hours using only 3uL of sample per well, this method provides a rapid and efficient way to assess the % full status of AAV samples.



466 A Platform for Interrogating Transgene Silencing and Epigenetic Signatures of AAV Genomes

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Loss of transgene expression following administration of AAV-based gene therapies is a well-established clinical challenge. For instance, clinical studies investigating AAV gene therapies for hemophilia have demonstrated an anti-capsid T-cell response, elevation of liver enzymes, and subsequent loss of therapeutic transgene expression. Separately, non-human primate studies have confirmed the number of vector genomes in the liver is far in excess of the number of transduced hepatocytes, potentially attributable to host factors exerting transcriptional silencing. However, mechanisms underlying the loss/silencing of expression remain elusive. Here, we report the use of CRISPR/Cas9-based activators and repressors to demonstrate the upregulation and downregulation of different promoter driven transgenes *in vitro*, respectively. We observe a significant decrease in gene expression and a modest increase in gene expression when targeting the inverted terminal repeats of the AAV genome using Cas9-KRAB and Cas9-VP64 fusions with their corresponding guide RNAs, respectively. In addition, data pertaining to interrogation of different host proteins and their potential roles in altering epigenetic signatures on AAV genomes will be presented. The overarching goal of these studies is to develop a roadmap for understanding the molecular mechanisms dictating silencing of AAV vector genomes and implications for clinical gene therapy thereof.

467 A Robust Machine Learning Algorithm for Improving AAV Capsid Performance

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Machine learning (ML) methods have shown promise in designing AAV capsids. Current methods typically fall under one of two

paradigms. The first relies on searching directly in the protein sequence space to find high-performing capsids using a regression model which maps sequences to target properties. These methods benefit from explicitly maximizing the desired property but are susceptible to pitfalls such as finding many false positives. The second paradigm sidesteps optimization by drawing protein samples directly from a generative model that learns a representation of functional AAVs and tries to generate similar capsids (e.g., Variational Autoencoders (VAE)). These methods generate capsids with an above-baseline functionality but don't typically produce high-performing samples, as that was not their original objective. Here we propose and experimentally validate VAEprop, a method combining the benefits of both approaches, which improves transduction more effectively than generative modeling or by searching in protein sequence space alone. To do this, we augment a traditional VAE's low-dimensional continuous representation by jointly training it with a regression model that maps the learned representation to the target property. The joint training procedure learns a meaningful and rich representation in which protein sequences with similar property measurements and sequence-level characteristics map to nearby points, but proteins with different measured properties map far from each other, even if the sequences contain shared motifs. By doing so, VAEprop transforms input sequences, which are tricky to optimize directly, into a continuous space better suited for optimization. After training the model on experimental data, we use it to find new points in the continuous learned representation space with a high predicted property value under the regression model. These points are then remapped to generate novel capsid sequences using the VAE. Critically, to improve on previous methods, we develop a novel stopping criterion that prevents our optimization from trailing off into regions far from the training data using the knowledge of the VAE. Our results show that our method is less likely to generate non-functional capsid sequences. We apply VAEprop to optimize transduction by designing thousands of variants of wild-type AAV9 comprising mutations in variable loop regions of VP3. Our validation assay targets the transduction of HEK293T cells in culture, as the shorter time between sequence design and capsid characterization enabled us to assess algorithmic performance quickly. In this setting, we compare VAEprop to two baseline methods by designing sequences using each method. The first samples sequences from a VAE, and the second optimizes sequences using a genetic algorithm (direct optimization). We show that VAEprop improves upon both methods. VAEprop successfully designs capsids at edit distances to wild-type of up to 16, of which almost half package. In comparison, only around a quarter of variants designed by the genetic algorithm successfully package. Furthermore, VAEprop's best-performing capsids transduce cells 16-fold better than AAV9 and between 2-4 fold better than the competing baselines. Most notably, the advantages offered by this method over baselines are equally applicable to in-vivo settings for capsid design, enabling effective optimization of capsid properties with high therapeutic relevance.

468 A Modular, Antibody-Based AAV Retargeting Platform for Efficient and Specific *In Vivo* Gene Delivery

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Adeno-associated virus (AAV) is a leading viral vector used in gene therapy to treat human diseases. Although AAV has many advantages as a gene therapy vector, one of the drawbacks to its use in systemic gene delivery, particularly for disorders that manifest in multiple organ systems, is the tendency to preferentially transduce the liver and require exceptionally high vector doses to effectively transduce peripheral tissues. Several safety concerns associated with high dose systemic AAV therapies have emerged in recent years, highlighting the need for more efficient AAV vectors that can achieve efficacy at lower doses. Although capsid library-based selection approaches are a powerful method to identify novel capsids with desired properties, the translatability of such capsids between species remains a challenge. Thus, we sought to develop a species-agnostic, modular AAV retargeting platform that utilizes rational capsid engineering and monoclonal antibodies to redirect viral particles to target cells. To accomplish this, we are using a protein tagging system to covalently couple antibodies to surface-exposed variable loops on the viral capsid. The tropism of the conjugated capsid can be further modified or abolished through the introduction of point mutations in the amino acid residues that serve as primary determinants of vector tropism. We have successfully used this approach to retarget multiple AAV serotypes to specific cell types *in vitro* and *in vivo,* using the cellular target of the retargeting antibodies to drive the specificity of infection. To establish the antibody retargeting platform and optimize it for *in vivo* applications, we focused on two target tissues with a clear need for better systemic delivery: skeletal muscle and central nervous system (CNS) targeting via efficient blood brain barrier (BBB) crossing. For muscle targeting, we identified a protein, CACNG1, that is specifically expressed in skeletal muscle. For BBB crossing, we chose Transferrin receptor (TfR), which is known to play a role in transcytosis of cargo across the BBB. Using panels of monoclonal antibodies raised against these target proteins, we demonstrate that AAV particles can be retargeted to skeletal muscle and can efficiently cross the BBB in an antibody-dependent manner *in vivo*. Retargeting can be achieved using bivalent antibodies, fabs, and scFvs, and using multiple AAV serotypes. Retargeted capsids display enhanced on-target delivery and marked liver detargeting compared to AAV9, a standard wildtype capsid used for muscle delivery and BBB crossing in human clinical trials. To demonstrate the cross-species translatability of our platform, we show that our antibody-targeted muscle-specific capsids outperform AAV9 when compared head-to-head in non-human primates using a barcoded library approach. Importantly, our retargeted capsids demonstrate enhanced delivery of therapeutic transgenes and improved functional rescue in mouse models of disease compared to standard

wildtype serotypes. Taken together, our modular antibody-based AAV retargeting system represents a novel platform for translatable, targeted gene delivery that holds promise for accelerating the development of effective gene therapies for a variety of human diseases.

469 Long-Read Sequencing of Diverse AAV *cap* Libraries through Barcode-Labeled Short Reads (BLaSR)

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Directed evolution is a high-throughput approach for creating and identifying novel AAV variants with enhanced properties, such as infectivity, targeted delivery, tissue spread, or immune evasion. The ability to deeply sequence these libraries offers insight into the evolutionary process, as well enable earlier identification of effective variants; however, accurate characterization of these diversified AAV libraries is not possible due to limitations in current sequencing technologies. Short read next-generation sequencing has a relatively low error rate and can capture variant mutations in which mutations are restricted to a specific region or within the read length, yet libraries diversified via methods such as error-prone PCR or random shuffling, in which diversity is spread through the full *cap* ORF, are unable to be correctly sequenced as individual clones. Long read sequencing offers this potential, but at substantially lower read depth and high error rates (1-15%). Here, we have made progress in addressing the limitations of both long- and short-read sequencing methodologies to profile a diverse AAV *cap* (~2.2kb) library. We demonstrate a new synthetic long-read sequencing methodology we term Barcode-Labeled Short Reads (BLaSR) that can sequence a diverse AAV *cap* library using a randomly-barcoded Tn5 transposon system. We harness the random and unbiased mechanism of the Tn5 transposon to insert random barcodes into the AAV *cap* library such that each *cap* variant contains insertions with a unique, identifiable barcode. This allows for the generation of barcode-linked next-generation sequencing short reads in which reads with the same barcode can be traced to the same AAV variant and thus be assembled into the longer *cap* variant sequence. We have engineered a robust pipeline to ensure highly efficient barcoded-Tn5 insertion into the AAV genome and have assembled thousands of AAV *cap* variants with an average 20-40x read depth, with a path towards considerably larger numbers of sequenced *cap* variants per run through further optimizations. Moreover, the use of BLaSR is not limited to AAV *cap* libraries; rather, this long-read sequencing methodology can be applied to other diverse DNA libraries that cannot otherwise be accommodated by next-generation sequencing.

470 High-Throughput AAV Screening Platform on Retinal Organoids to Target Cell Types

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Targeted gene delivery to only disease-critical cell types greatly enhances the therapeutic potential of adeno-associated virus (AAV)-mediated gene therapy and reduces undesirable side effects. This can be achieved by placing a specific on-off switch for gene expression - a promoter - in the viral genome. Since the precise mechanisms of cell type specific expression are not yet understood, many promoter variants need to be tested to find the ideal expression profile to treat a particular disease. Currently, human retina explants from post-mortem donations are used for individual testing of AAV promoter variants, but limited availability and significant labor constrain the number of variants that can be compared. Human retinal organoids closely resemble most cell types of the human retina and can be generated in large amounts. The semi-automated platform we developed uses the more available model system of retinal organoids to pre-screen promoter variants in AAVs with higher throughput. Our pipeline starts with cloning of AAV plasmids harboring promoter variants driving GFP expression in a 96-well plate format, followed by high throughput production of AAVs on a small scale sufficient to infect retinal organoids. High-resolution 3D live imaging of infected organoids is applied to evaluate the AAV promoter variants for expression in the therapeutically most relevant cell types. After quantitative image analysis, screened promoter variants are ranked for further testing on human retina explants. Besides speeding up the discovery of new cell-type targeting promoter sequences for gene therapy, this will result in new tools for understanding retinal diseases using organoids as a model.

471 A Robust and Flexible Baculovirus-Insect Cell System for AAV Vector Production with Improved Yield, Capsid Ratios and Potency

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Manufacturing of recombinant adeno-associated viruses (rAAV) for gene and cell therapy applications has increased significantly and spurred development of improved mammalian and insect cell-based production systems. We developed a baculovirus-based insect cell production system- the Sangamo (SGMO) Helper- with a novel gene architecture and greater flexibility to modulate the expression level and content of individual Rep and Cap proteins. In addition, we incorporated modifications to the rAAV6 capsid sequence that improves yield, capsid integrity and potency. Production of rAAV6 using the SGMO Helper had improved yields compared to the Bac-RepCap helper from the Kotin lab. SGMO Helper-derived rAAV6 is resistant to a previously described proteolytic cleavage unique to baculovirus-insect cell production systems and has improved capsid ratios and potency, *in vitro* and *in vivo*, compared to rAAV6 produced

using Bac-RepCap. NGS sequence analysis demonstrated that the SGMO Helper is stable over six serial passages and rAAV6 capsids contain comparable amounts of non-transgene DNA as rAAV6 produced using Bac-RepCap. AAV production using the SGMO Helper is scalable using bioreactors, with similar improvements in yield, capsid ratio and *in vitro* potency. Our studies demonstrate that the SGMO Helper is an improved platform for rAAV manufacturing to enable delivery of cutting-edge gene and cell therapies.

472 Rational Engineering of the AAV P5 Promoter Improves Recombinant Vector Purity

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Previous work has demonstrated that both P5- and ITR-associated nucleic acid contaminants from plasmids used to produce recombinant adeno-associated virus (rAAV) vectors can be expressed *in vivo*, highlighting a need to further characterize their implications and develop strategies to increase vector purity. These sequences are encapsidated within fully functional virions, resulting in transcription and translation of undesired plasmid-derived sequences. We hypothesized the packaging of contaminant plasmid sequences is related to the P5 promoter structure and Rep gene expression during production, and that by manipulating critical elements of the P5 promoter and modulating Rep expression, we can reduce the encapsidation of these contaminant sequences. To prevent this encapsidation, we explored the use of homologues from alternative serotypes as replacements for the commonly used P5 promoter in rAAV production. Our data indicate that the use of homologous or rationally designed Rep protein Binding Elements (RBE) and Terminal Resolution Sequence (TRS) loop modifications to the P5 promoter can reduce encapsidation of nearby DNA by roughly ten-fold. Additionally, we found that replacing the canonical AAV2 P5 promoter with alternative homologues can reduce the frequency of encapsidation of contaminants from distant hotspots by over 20%. A subset of these surrogate promoters also improves viral titer by 30-40% during small scale rAAV production in a transgene agnostic manner. Meanwhile, replacing the P5 promoter with a non-related viral promoter, results in aberrant Rep expression, low titers, and high contamination. Rep westerns and fluorescent reporter assays highlight changes in promoter activity correlated with changes to vector titer and purity. These assays were used to identify the implications of promoter modifications on promoter function. Modifications which improved purity and had the most detrimental effect on viral titer, had the strongest impact on promoter activity. Specifically, we observed decreased large Rep expression and increased small Rep expression. We then found that additional modifications to compensate for altered promoter activity rescued viral titer, suggesting these assays may be relevant in establishing and optimizing conditions for rAAV production. These modified promoters may prove to be viable alternatives to the canonical AAV2 P5 routinely used in rAAV production. Large scale production, vector characterization and *in vivo* assays with these new AAV promoters are currently underway. In conclusion, rAAV contamination can be reduced by rationally engineering the P5 promoter. Our findings have implications for making safer, purer products for rAAV gene therapy.

473 Human Bocavirus 1 Muscle Transduction Efficiency Following Local and Systemic Injections in a Murine DMD Model

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Adeno-associated virus (AAV)-mediated gene therapy is the leading approach to treating various human diseases. However, certain gene editing and replacement strategies are unviable due to the limited ~4.7kb packaging capacity of AAV. Human Bocavirus (HBoV)-mediated gene therapy is a relatively new alternative with an advantageous ~5.5kb packaging capacity. Here, we evaluated the local and systemic muscle transduction efficiency of the HBoV1 vector in mdx mice, a murine Duchenne muscular dystrophy model. The HBoV1 reporter vector expressed green fluorescent protein (GFP) under the transcriptional regulation of the cytomegalovirus promoter. The same vector genome was packaged in AAV8/9 for comparison. Local delivery was studied by intramuscular injection to the tibialis anterior (TA) muscle (n=4 mice). The HBoV1-GFP vector was injected into the left TA muscle at the dose of 3.80E10 vg/muscle. The right TA muscle received the AAV8/9-GFP vector at the dose of 0.24E10 vg/muscle, 0.95E10 vg/muscle, 3.80E10 vg/muscle, or 7.60E10 vg/muscle. Systemic delivery was studied by tail vein injection at the dose of 3.80E11 vg/mouse (n=2 mice). Tissues were harvested at four weeks post-injection. For the local injection study, we detected robust GFP expression in muscles that received 0.95E10 vg/muscle, 3.80E10 vg/muscle, and 7.60E10 vg/muscle of the AAV vector. GFP expression was not detected in the muscle that received 0.24E10 vg/muscle of the AAV vector. Weak GFP expression was observed in one HBoV1 vector-injected muscle. No GFP expression was detected in the remaining three HBoV1 vector-injected muscles. Vector genome copies were quantified with quantitative real-time PCR. We found a correlation between GFP expression and vector genome copy number in both AAV and HBoV1-injected muscle. Overall, for the same dose, the vector genome copy number was substantially lower in the muscles that received the HBoV1 vector. We are currently processing and evaluating the various tissues from the intravenously injected mice. We will report our findings at the 26th annual meeting.

474 The Effect of Lithium on Viral Vector Production and Gene Delivery

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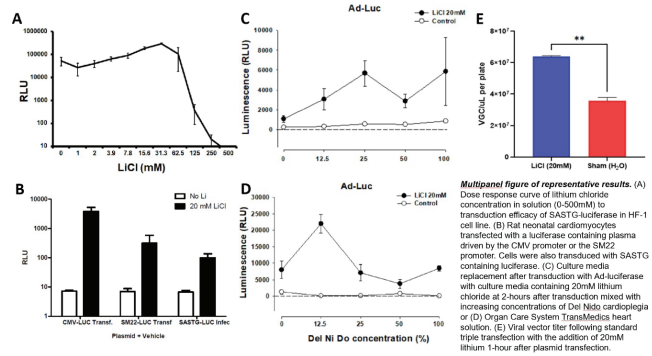
Background. Viral vector yield and potency of viral vectors remain significant issues that limit the manufacturing of gene therapies and maintain the costs of production exceedingly high. A number of chemicals have been reported to improve viral vector production and gene delivery. Here we investigate the effects of lithium (Li), a widely used drug for treating mood disorders, whose major effect is through inhibition of glycogen synthase kinase-3. We characterize the addition of Li at various steps of viral vector production and transduction: plasmid production, triple transfection for viral vector production, gene delivery by vector transduction or plasmid transfection. ***Methods.*** Plasmids used for AAV production were isolated from *E. coli* using the PureYield Plasmid Maxiprep System (Promega, WI). Li was added into the bacterial culture 3-hours prior to plasmid isolation and DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher, MA). Recombinant Adeno-associated viral vectors (rAAV) were generated by standard triple transfection. rAAV titers were measured using quantitative PCR (BioRad, CA). The Adenoviral (Ad) vector was obtained from the Pittsburgh Human Gene Therapy Center (Pittsburgh, PA) and expanded in our lab. Various cell types were plated on 96-well plates at 10,000 cells per well and were transduced at 24-hours after plating with either 1,000 Ad viral particles per cell or 10,000 rAAV particles per cell. 293 cells were transfected with plasmids driven by either the cytomegalovirus (CMV) or smooth muscle 22 (SM22) promoter using either polyethylenimine or Fugene 6 (Roche, Switzerland). Two-hours following either vector transduction or plasmid transfection, Li was added, and luciferase activity was assessed via luminometry 48 hours later. For some of the viral vector transduction experiments, a mixture of culture media with cardioplegia solution (Del Nido solution, Baxter Inc, IL; Organ Care System heart solution, TransMedics, MA) was used during the transduction period. Luciferase reagent (ONE-Glo, Promega, WI) was added to the cells and luminometry was performed with a Veritas luminometer (Turner Biosystems, CA). ***Results.*** Luciferase activity increased 3-5-fold when 20mM of LiCl was added at 2-hours post-transduction of HeLa cells, HF-1 cells, and rat neonatal cardiomyocytes with AAV 1, 2, 5, 6, and SASTG viral vectors. Luciferase activity also enhanced 532- and 44.7-fold for CMV-luciferase and SM22-luciferase plasmids compared to experiments in the absence of lithium., respectively. Addition of lithium did not enhance plasmid yield from bacteria. Addition of 20mM Li led to a significant 1.8-fold enhancement in the number of rAAV particles produced. Transgene expression using Ad vectors were improved by LiCl in human aortic tissue and 20mM LiCl addition 2-hour post transduction improved transgene expression of the Ad vectors that was originally suppressed by cardiac perfusion solutions. ***Conclusion.*** We describe that Li has enhancing effects in different steps of viral vector production and gene delivery. Given its low cost and already FDA approved status for clinical use, it is an attractive molecule for implementing in widespread viral vector production and gene therapy delivery. We describe consistent upregulation of gene expression in eukaryotic cells provided plasmid, AAV, or Ad regardless of type of cell or promoter used. unknown.



Multipanel figure of representative results. (A) Dose response curve of lithium chloride concentration in solution (0-500mM) to transduction efficacy of SASTG-luciferase in HF-1 cell line. (B) Rat neonatal cardiomyocytes transfected with a luciferase containing plasma driven by the CMV promoter or the SM22 promoter. Cells were also transduced with SASTG containing luciferase. (C) Culture media replacement after transduction with Ad-luciferase with culture media containing 20mM lithium chloride at 2-hours after transduction mixed with increasing concentrations of Del Nido cardioplegia or (D) Organ Care System TransMedics heart solution. (E) Viral vector titer following standard triple transfection with the addition of 20mM lithium 1-hour after plasmid transfection.

475 Advanced Dual AAV Vector-Intein Mediated Systems: Improving Dual Vector Doses

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Background and novelty: Adeno-Associated Viral (AAV) vectors are one of the most well-established vectors used in the clinic for *in vivo* gene therapies. Despite significant improvements since their development, AAV vectors still present several limitations. One major bottleneck is the small packaging size restricting its use to treat diseases requiring the delivery of large genes. To overcome this, dual AAV vector delivery systems relying on protein trans-splicing can be applied to reconstitute large proteins, being the split-intein DnaE sequence from *Nostoc Punctiforme* (Npu DnaE) currently the most used. However, it presents low reconstitution efficiencies, demanding higher vector dose administration to attain efficient therapeutic effects. Low-quality vector preparations can also contribute to limited gene reconstitutions by promoting vector competition and inhibiting transduction. This work seeks to overcome inefficient protein reconstitution rates and decrease vector doses of current dual AAV vector-intein-mediated systems by applying split-inteins with unmatched trans-splicing rates combined with higher-quality AAV vectors. **Experimental approach:** Studies were conducted to evaluate the performance of engineered-consensus (Cfa) and cyanophage-like Gp41-1 split-inteins when compared to the Npu DnaE split-intein. AAV expression cassettes were developed to encode frGFP terminal halves fused to split-inteins. Full-length frGFP controls containing intein a.a. remaining (Scar-frGFP) after split-intein reaction were developed. Protein reconstitution was assessed by transient transfection and dual-AAV co-transduction. Impact of AAV vector quality (percentage of empty and full particles) on dual-AAV co-transductions was assessed. An upstream-to-downstream process was performed with and without a full particle enrichment step (using anion exchange chromatography). Dual-AAV co-transductions using low AAV vector doses are under evaluation. **Results and discussion**: Transfection studies showed a 2-fold decrease in fluorescence intensities with Scar-frGFP control constructs when compared to non-scared frGFP, revealing that intein residues impact frGFP protein function. Cfa and Gp41-1 presented over

2-fold higher reconstitution rates than NPU DnaE and almost 100% frGFP reconstitution. Dual-AAV2 co-transductions were performed to determine optimal transduction conditions by testing different vector doses ($1x10^4$-$5x10^4$ V.G./cell). Results demonstrated 40% decrease in fluorescence intensity compared to single transductions, suggesting vector competition. Initial Cfa and GP41-1 split-inteins dual-AAV co-transductions resulted in 10% more frGFP reconstituted positive cells than Npu DnaE. The established AAV vector full particle enrichment step increased over 3-fold the quality of the viral preparations. On average, 60-75% of full AAV particles were obtained in all AAV2 split-intein productions. Dual-AAV2 co-transduction studies with different vector doses are currently undergoing, to determine the impact of viral vector quality with optimized split-inteins. This work showed improved protein reconstitution efficiencies, revealing Cfa and Gp41-1 as promising split-inteins for dual AAV delivery. Further developments combining these split-inteins with enhanced vector qualities will enable the reduction of AAV dose in therapeutic administrations, paving the way for the delivery of large therapeutic transgenes.

476 Novel mRNA Delivery System Based on Positive-Sense Single-Stranded RNA Viruses

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Background: The CRISPR (clustered regularly interspaced short palindromic repeats) gene editing technique has been extensively exploited in numerous hereditary illnesses. However, limitations in cargo size, off-target effects, and insertional mutagenesis prevent it from being used *in vivo*. Transferring mRNA could solve these issues, as mRNA delivery systems are one of the most promising approaches to transfer genes of interest.Methods: We created a non-replicating mRNA viral vector based on the Semliki Forest virus (SFV). Several cell lines, including those generated from human, mouse, and hamster, were used to confirm the effectiveness. We used Ai9 mice to show the new vector's biodistribution. Neutralizing antibodies, blood chemistry, and histology of various administration routes were also tested to investigate the immunological responses to high-dose injection.Results: We developed a non-replicating viral mRNA delivery method based on the SFV. This new displayed remarkable transduction efficiency in various cell lines. The viral particles distributed in livers, spleens, skeletal muscles and brains via intravenous injection and efficiently delivered into the central nervous system via intracerebroventricular injection. Little inflammation was seen in response to high-dose treatment in both the histology and blood chemistry. Similar to other viral vectors, neutralizing antibodies were discovered one week after injection and decreased over time. Conclusions: We created an mRNA delivery method with excellent transduction efficiency and little immunogenicity. We confirmed the efficacy in multiple cell lines and the biodistribution in Ai9 mice and exhibited the immunological responses of several administration routes. This innovative mRNA vector offered a promising option for RNA therapy.

477 A Comprehensive Characterization of Full, Partial, and Empty Lentiviral Vectors

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Lentiviral vectors are biological vehicles used to deliver therapeutic genes ("payload") into cells. The characterization of lentiviral vectors is challenging due to their complex physical structure. To be considered functional, lentiviral vectors require at least three critical components: 1) An outer envelope, comprised of VSV-G protein 2) A capsid housing the genetic material, comprised of p24 protein and 3) The payload itself constituted by the therapeutic gene. Since there is no assay currently on the market that measures all three attributes, scientists must piece together data from several different assays to understand the composition of the vector, which is often laborious and unreliable. Currently, a microarray chip-based assay on the market can offer a partial characterization of lentiviruses. However, this assay does not provide any information on the presence or absence of the payload. This is an integral piece of information which would determine the functionality of the lentiviral vector. In this study, we developed a new characterization method that selectively targets VSV-G, p24 and the RNA payload by using a permeant fluorescent nucleic acid stain [a]. Using this method, we can quickly visualize and quantitate the lentiviral vector at three separate levels for a comprehensive understanding of lentiviral vector products. Importantly, this method can enable new vector design and process optimization to enrich full lentiviral vectors.
[a] Patent pending.

478 Improved *In Vivo* Lentiviral Gene Therapy of Chronic Granulomatous Disease

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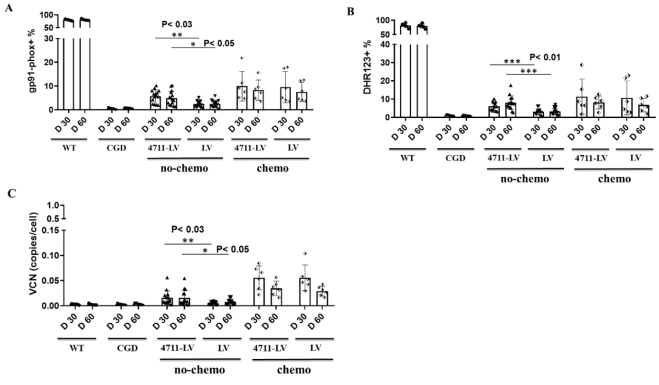
Background: Chronic granulomatous disease (CGD) is a congenital immunodeficiency characterized by severe life-threatening infections. CGD patients lack reactive oxygen species (ROS) in phagocytic leukocytes and often develop widespread tissue granulomas. Gene therapy based on autologous hematopoietic stem cell transplantation (HSCT) is a promising treatment for CGD, but the poor tolerance of CGD patients to transplantation-related chemotherapy presents a major limitation to this strategy. "*In vivo*" gene therapy is based on direct delivery of transgenic vehicles into patients, which could overcome the difficulties in HSCT. The standard VSVG-pseudotyped lentiviral vector (LVs) have been shown ineffective for direct *in vivo* injection, since VSVG is rapidly inactivated by human complement and innate immunity. We have previously demonstrated LV-*CYBB* (cytochrome B-245 beta chain, or p91-phox) expression by universal or myeloid-specific promoter through *ex vivo* HSCT gene therapy could correct CGD phenotype in X-CGD mice (DOI: 10.1089/hum.2022.140). Here we report an improved direct *in vivo* LV gene delivery strategy based on minimal immune suppressive conditioning and an innovative major histocompatibility complex (MHC) knock down LV system to increase intravenous (iv) LV gene therapy

efficacy. **Methods:** To increase iv LV gene delivery efficiency, X-CGD mice (X-CGD; B6.129S-Cybb^{tm1Din}/J) were pre-treated with chemo-conditioning (busulfan 5 mg/kg), cyclophosphamide 100 mg/kg and dexamethasone 5 mg/kg). In addition, the 293T-based LV packaging cells were modified with cytomegalovirus-based MHC-knock down genes (US11 and ICP47) and innate immune escape protein CD47 (4711-LV). The CGD mice were iv injection of 2x10^9 transduction units (TU) 4711-LVs carrying a myeloid-specific miR223 promoter driving the expression of *CYBB*. The transgene expression, vector copy number (VCN), and CGD phenotype correction were investigated. **Results:** We compared *in vivo* LV gene therapy in CGD mice with or without chemo-conditioning, using regular LV-*CYBB* or 4711-LV-*CYBB.* The expression and function of the LV-*CYBB* were analyzed by intracellular gp91-phox staining and DHR123 assay 4 and 8 weeks after iv gene delivery. Based on flow cytometry, we detected gp91-phox positive cells in the blood after 4 and 8 weeks as the following, no-chemo-4711-LVs: 1.98-10.22% and 1.34-10.24%, no-chemo-LVs: 0.6-5.14% and 0.26-5.87%, chemo-4711-LVs: 4.76-21.8% and 3.61-15.6%, chemo-LVs: 3.56-17.95% and 3.65-12.59%, as compared with WT mice: 76.5-84.2% and 78.98-86.12% (Fig. A). Functional analyses of ROS showed the following, no-chemo-4711-LVs: 3.1-10.09% and 2.65-17.53%, no-chemo-LVs: 0.25-6.21% and 0.81-6.99%, chemo-4711-LVs: 1.74-28.73% and 2.61-12.6%, chemo-LVs: 2.78-22.95% and 1.98-11.59%, as compared with WT: 70.19-91.86% (Fig. B). The LV VCN in the peripheral blood was detected by qPCR as the following, no-chemo-4711-LVs: 0.58-5.61% and 0.26-5.4%, no-chemo-LVs: 0.12-0.93% and 0.18-1.75%, chemo-4711-LVs: 2.22-8.4% and 1.62-5.6%, chemo-LVs: 2.96-10.39% and 1.66-4.27% after 4 and 8 weeks, respectively (Fig. C). The results supported that the use of 4711-LVsincreased *iv* LV gene delivery efficiency even without chemo-conditioning.**Conclusion:** We have developed a novel 4711-LV system to improve direct iv LV gene delivery without chemo-conditioning. This new strategy could potentially improve *in vivo* LV gene therapy applications.



479 A Nonclinical Method for Identifying an Anti-Drug Antibody Response Against an Oncolytic Virus Therapeutic

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Introduction: Oncolytic vectors (OVs) as a novel therapeutic modality create unique challenges for anti-drug antibody (ADA) assay development; there is limited guidance for assessing immunogenicity, the drug is complex and contains multiple viral proteins that are immunogenic, and assay development may pose high material demand requirements. Here, we present a nonclinical assay to detect and characterize the presence of ADA against a therapeutic OV. **Methods**: To identify the presence of ADA, a plate-based screening and confirmatory assay was developed. Whole virus was used as the coating material to capture ADA. The positive control was a polyclonal antibody generated from rabbits immunized with OV. Commercially sourced naïve mouse serum was used to determine the cut points of the assay and identify positive individuals. Samples consisted of serum collected from Balb/C mice dosed once with therapeutic OV and serum collected from Balb/C and C57Bl/6 mice dosed three times with therapeutic OV. Alternative antibodies against viral proteins were interrogated as positive controls and although not selected, they provided insight into the condition of the virus bound to the plate. The immune response against viral proteins is confirmed through Western blot using immunized mouse serum as the probing material and recombinant proteins and immunogen as loading materials. **Results**: An ADA assay was successfully developed to determine the presence of ADA against OV using naïve and OV-dosed mouse serum samples. Using the developed ADA assay, it was determined Balb/c mice generated ADA against OV after a single dose. In a separate Balb/C and and C57 mouse study, upon repeat dosing (a total of three doses), ADA was also detected at relatively similar levels as the single dose study. Western blot data confirmed an ADA response against the viral proteins N, M, and GP. Finally, we demonstrate, using monoclonal antibodies to the viral nucleoprotein and matrix protein, that positive signal is obtained using whole virus, suggesting that these antigens may be present on the exterior of the viral membrane as well. **Conclusions**: Our assay demonstrated an approach to identify treatment emergent ADA response against a novel OV therapeutic. Although it is not feasible to identify pre-existing ADA in laboratory mice that would not have prior exposure to OV, it is anticipated that pre-existing ADA in humans would be detectable with the clinical version of this assay due to the sensitivity of the assay. This assay would allow for the ability to determine presence/absence of pre-existing ADA prior to enrollment of a patient to a clinical trial and to provide insight into drug efficacy after repeat dosing.

480 Discovery Platform for Identification of Novel Cell Type Specific Synthetic Promoters for Gene Therapy Vectors

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The goal of gene therapy is to be able to achieve strong and very specific expression of a therapeutic gene. Most fundamental control of gene expression takes place at the promoter region which controls the transcription of a gene. For gene therapy vectors, viral promoters can be ubiquitously and constitutively active, which may lead to abnormally high cellular levels of a therapeutic transgene. Conversely, host cell promoters, though tissue-specific, may only provide low level gene expression and are typically of considerable size, not optimal for use in viral vectors. Lack of promoter optimisation may lead to transgene toxicity, unwanted immune activation and/or therapeutic failure. Promoter engineering provides unique opportunities to improve clinical performance of the viral vector and thereby improve the safety of gene therapies. Here, we have developed a strategy for synthetic promoter design, based on in-house-generated transcriptomic data, epigenetic datasets and bioinformatic sequence analyses. Using either known or de novo identified native cis-regulatory elements functional in target tissues, combinatorial synthetic promoter libraries were designed to allow massively parallel activity screens. We sought to use RNAseq-based screening of barcoded transcription units to identify novel synthetic promoters and determine the relative frequency and transcriptional activity of these promoters in specific target cell populations. Champion promoters demonstrated robust cell- or tissue-specific patterns of expression (~10-100 times stronger in target cells) and transcriptional activity varied dramatically between promoters (<10-fold difference). Our data indicate that rational promoter design coupled with suitable screening campaigns can be valuable tools for improving the performance of viral vectors and the on-target safety of gene therapies.

481 Single-Cell Lentiviral Vector Integration Sites and Clonal Tracking Assay for Cell and Gene Therapy

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Lentiviral vector (LV) has been widely adopted as an efficient vehicle to deliver transgenes into cells due to its long-term efficacy. However, the semi-random integration of LV has raised safety concerns due to its potential to trigger tumorigenesis during CAR-T therapy. To mitigate this issue, characterization of vector integration on clonal expansion after gene therapy has become a crucial practice to monitor the activity of retroviral vectors on in vivo selection of patient clones. Here, we developed a single-cell resolution, lentiviral vector integration site assay to survey the co-occurrence of specific integration sites with somatic genomic variants. Based on a set of LV transduced cell lines with known integration sites validated by orthogonal data, a targeted panel was designed to cover 5' and 3' ends of each integration site with predefined integration orientations. Samples include negative control and LV transduced cell lines with known vector copy numbers ranging from one to four copies. Using a titration experiment design, we demonstrate the capability to quantitatively detect individual cells harboring specific vector integration sites and longitudinal tracking of cell clones with different vector copy numbers. Furthermore, another set of 99-plex human genome amplicons are included to characterize somatic variants in the cell population of a sample. Taken together, a high throughput single-cell multi-omics platform enabled us to simultaneously identify somatic variants along with vector integration events in individual cells, providing both potential functional mutation identification and clonal tracking capabilities. The development of single-cell lentiviral vector integration sites and clonal tracking assay provides a unique opportunity to better study longitudinally CAR-T cell clonal expansion and lead to a more effective therapeutic agent.

482 *APOE*-Targeted Epigenome Therapy for Late Onset Alzheimer's Disease

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Background: There is an urgent need to shift the paradigm of Alzheimer's disease (AD) drug discovery to the development of new targets. *APOE* is the strongest and most reproducible genetic risk factor for late-onset AD (LOAD). Moreover, 50% reduction in *APOE* levels showed beneficial effects in AD cellular and mouse models. Thus, *APOE* gene holds promise as a potential therapeutics target for LOAD. Here, we developed an epigenome therapy platform to reduce *APOE*e4 expression by targeted modification of the epigenome landscape across *APOE* locus. **Methods:** We developed epigenome therapy based on CRISPR/deactivated(d)Cas9 editing technology fused with a repressor molecule and delivered by viral vehicle. We designed a set of gRNAs to target regulatory elements in *APOE* region and within exon 4 overlapping the SNP that defines the *APOE*e4 allele. We validated our epigenome therapy platform *in vitro* using human induced pluripotent stem cell (hiPSC)-derived models and *in vivo* using mice. **Results:** The viral dCas9-repressor vector showed a robust decrease, ~50%, in *APOE*-mRNA levels in hiPSCs and the derived cholinergic neurons, microglia, and organoids models. The system specifically targeted the *APOE*e4 allele exhibiting the reduction effect in all hiPSC-derived cellular and organoids models with the e4-allele while there was no effect in the *isogeneic* hiPSC-derived models homozygous for the e3-allele. Further examination of the transduced e4/4 organoids demonstrated that the specific reduction in *APOE*e4 expression led to lower Aβ42/40 levels. Moving onto *in vivo* studies, we stereotactically injected the viral dCas9-repressor vector into the mice hippocampus. The results showed a significant decrease amounting to ~70% in mouse endogenous *Apoe* expression, demonstrating a strong and specific repression effect *in vivo*. **Conclusions:** Collectively, our results provided *in vitro* and *in vivo proof-of-concept* for the utility, efficacy, and specificity of the *APOE*-targeted epigenome therapy. Our epigenome therapy strategy for targeted fine-tuning of *APOE*e4 expression based on dCas9 technology is *translational* toward the development of a therapeutics approach to prevent and/or delay LOAD

onset. Furthermore, the technology offers the opportunity to refine the platform for the development of gene-specific and even allele- and cell-type- specific therapies, and by that enables the advancement of strategies for precision medicine in LOAD.

483 Automating the Analysis of Genomic Damage: Instability and Genotoxicity Using AI and Machine Learning

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Structural variants, such as inversions and translocations, are common by-products of cellular engineering processes such as CRISPR editing and insertion of transgenes. These variants are formed by misrepair of DNA and result in a heterogenous mixture of low-prevalence variants that involve edit-site, off-target and random DNA breakpoints. **d**irectional **G**enomic **H**ybridization™ (dGH™) is a unique cytogenetic technique for mapping the structure and structural variation of many individual genomes in single cells. Based on images of millions of DNA probes designed against a reference genome and hybridized to metaphase chromosomes, dGH assays provide true de novo, unbiased detection of structural variants as small as 2Kb anywhere in the genome. dGH is ideal for measuring these editing by-products as well as potentially genotoxic outcomes such as sub-clonal outgrowth, insertional mutagenesis and chromothripsis. While as few as 20 single-cell genomic maps can be sufficient to detect high prevalence structural variants, achieving sufficient detection power for the low prevalence (<1%), potentially genotoxic variants requires mapping of hundreds to thousands of single-cell genomes. Due to cost and turnaround time, reliance on manual counting limits dGH assay analysis to hundreds of cells per sample. In this study we demonstrate the automation of the entire dGH workflow using AI and machine learning techniques, removing this limitation and thereby making detection of ultra-low prevalence, potentially genotoxic variants practical and efficient.

484 *In Vivo* CRISPR Base Editing of Angptl3 via Lipid Nanoparticle Delivery

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Human dyslipidemia diseases, such as coronary artery disease (CAD) and Non-alcoholic fatty liver disease (NAFLD), are classified by an abnormally high-level of cholesterol and/or fats in the blood. *Angptl3* has been identified as a potential target of interest for treating dyslipidemia diseases as it encodes for angiopoietin-like 3 (ANGPTL3), an enzyme that regulates plasma lipoprotein levels. Loss of function (LOF) variants of *Angptl3* are naturally occurring with no known associated complications. Individuals with these mutations have shown reduced levels of plasma low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) and protection against CAD. Therefore, we rationalized that inducing a LOF in *Angptl3* may protect against these diseases. Utilizing a base editing approach, we package BE mRNA and a *Angptl3*-targeting sgRNA within a liver targeting lipid nanoparticle (LNP) to KO *Angptl3*. First, we formulated LNPs containing one of three lipids (306-012B, Lipid88 and Lipid10) by loading them with ABE8e and one of two different single guide RNAs (sgRNAs), sgRNA_SA or sgRNA_SD. We then injected them *in vivo* and collected the liver 7 days later. From extracted liver DNA and NGS we found that Lipid88 achieved the highest editing efficiency with both candidate sgRNAs. Next, to test the long-term efficacy, we injected mice and analyzed editing. After 7 days, we found that mice treated with LNPs achieved high editing efficiency in the liver (>60%) and limited editing in seven other organs (<2%) (Figure 1).

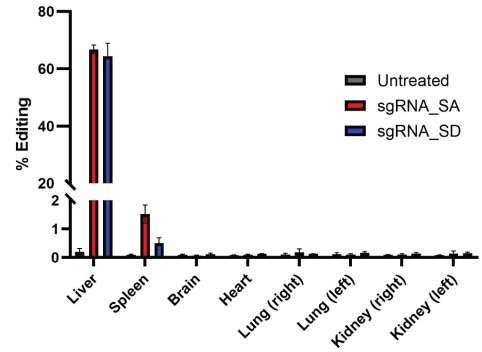

Figure 1. LNP biodistribution.

We also tested the therapeutic effect by performing analyzing serum levels of ANGPTL3, LDL-C, and TG. We observed a significant reduction in serum levels at day 7 and 30 using one or both of our sgRNAs compared to untreated mice (Figure 2).

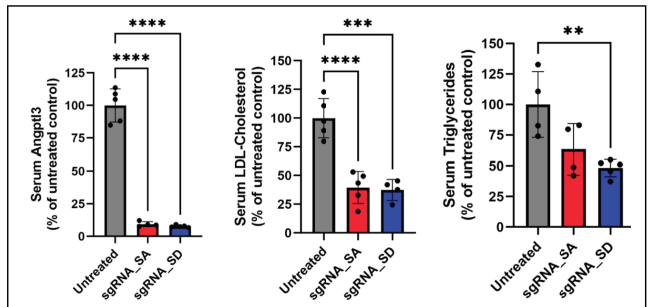

Figure 2. ANGPTL3, LDL-C, and TG levels after 30 days. Significance measured by one-way ANOVA.

Additionally, to assess the toxicity of these treatments, we used a higher dose (2 doses), proposing that a higher dose of our treatment would be more toxic than a lower dose (1 dose) and after 48 hours we collected blood and tested the levels of alanine transaminase (ALT), aspartate transaminase (AST), and interleukin-6 (IL-6). High levels of ALT and AST are associated with liver damage and high levels of IL-6 are associated with an increased immune response. We determined that there was no significant increase in toxicity associated with these treatments. In conclusion, the knockout of *Angptl3* has shown to lower levels of LDL-C and TG and protect against diseases such as CAD and NAFLD. It is of particular interest to see if this KO can additionally be used as either a treatment or prevention for NAFLD. This will be

done by analyzing the effect of our LNPs using both an obese mouse line known to develop NAFLD and a wild type mouse line on a high fat and cholesterol diet.

485 Evaluation of Humanized Mouse Models for *In Vivo* HSC Gene Therapy

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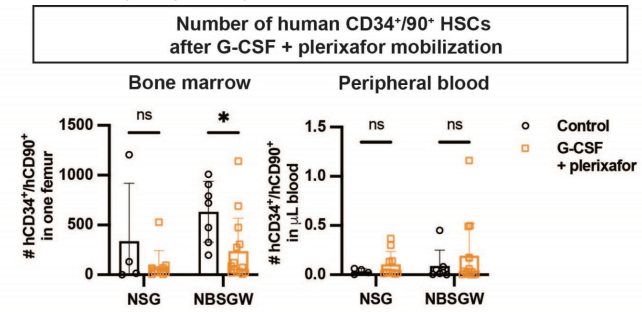
In vivo hematopoietic stem cell (HSC) gene therapy is a promising strategy that eliminates the need for burdensome leukapheresis, myeloablation, and transplantation. To preclinically test novel therapeutic reagents for in vivo gene therapy, humanized mouse models are invaluable tools. Three conditions must be satisfied to demonstrate effective in vivo gene therapy in humanized mouse models. First, the model should support high human HSC engraftment in the bone marrow (BM) so there are enough $CD34^+$ $CD90^+$ long-term HSCs (LT-HSCs) to target. Second, human HSCs in the murine BM need to become accessible to the therapeutic reagents introduced in vivo. HSCs in the BM are largely inaccessible to the reagents introduced in circulation and mobilization is considered the best way to make HSCs accessible. Lastly, human HSCs in mice should be permissive for the therapeutic agents such as viral vectors. Here, we comprehensively compared humanized NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) and NOD.Cg-*Kit*$^{W-41J}$ *Tyr* $^+$ *Prkdc*^{scid} *Il2rg*^{tm1Wjl}/ThomJ (NBSGW) strains for $CD34^+$ $CD90^+$ HSC engraftment, mobilization, and vector transduction in vivo. For this, we humanized irradiated NSG and non-irradiated NBSGW neonates with human umbilical cord blood-derived $CD34^+$ cells. First, we observed that the NBSGW strain supports significantly higher human $CD34^+$ $CD90^+$ engraftment in the murine BM compared to the NSG strain. Surprisingly, we found hundreds of $CD34^+$ $CD90^+$ HSCs in the peripheral blood (PB) of both strains, indicating that there is a low-level circulation of human HSCs in these humanized mouse models. Next, we treated humanized mice with granulocyte-colony stimulating factor (G-CSF) and AMD3100 (plerixafor) to assess HSC mobilization. Despite successful mobilization of human HSCs from the BM of NBSGW mice, there was no corresponding increase in the number of HSCs in the PB. Since mobilization was not successful in making human HSCs available in circulation, VSV-G pseudotyped lentivirus encoding GFP was directly injected into the BM. Although GFP-positive cells were found in the BM, none of the GFP-positive cells were $CD34^+$ $CD90^+$, indicating that the lentivirus could not efficiently target LT-HSCs. To investigate the reason for the inefficient in vivo viral transduction of human HSCs, we analyzed the expression of low-density lipoprotein receptor (LDL-R), a major entry port of VSV-G pseudotyped lentiviral vectors, on steady-state human $CD34^+$ $CD90^+$ HSCs from the mouse BM as well as from leukapheresis samples from G-CSF-treated human donors. In both cases, we found a very low-level expression of LDL-R indicating that steady-state as well as freshly isolated G-CSF-mobilized human HSCs have limited accessibility to VSV-G based viral vectors. From our findings, we concluded that NBSGW mice provide a better platform to test in vivo human HSC targeting strategies due to high LT-HSC engraftment. Our work also warrants for a development of VSV-G-independent gene therapy strategies such as adenovirus or measles virus to target HSCs in vivo. Another alternative is surface-engineered viral vectors for HSC-specific targeting. Considering the wide usage of humanized mouse models and HSC mobilization in the field, our work highlights the importance of identifying a proper model and method to study in vivo HSC targeting strategies.



486 Population-Wide Gene Disruption in the Murine Lung Epithelium via AAV-Mediated Delivery of CRISPR-Cas9 Components

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With the aim of expediting drug target discovery and validation for respiratory diseases, we developed an optimized method for in situ somatic gene disruption in murine lung epithelial cells via AAV-mediated CRISPR-Cas9 delivery. Achieving high editing efficiency on a populational-level in alveolar cell type II (AECII) and airway epithelium, e.g. club and ciliated cells, is of particular value given the roles these cell types play in cystic fibrosis, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis. We optimized parameters including AAV capsid serotype, route of administration, Cas9 variant, and AAV vector configuration driving Cas9 expression. We demonstrated feasibility and efficiency of sequential AAV intratracheal dosing with combinatorial guide RNA expression cassettes. Application of these optimized methodologies in a reporter mouse model (Rosa26-LSL-tdTomato) showed gene editing rates up to ~85% in AECII and distal airway cells. The most robust editing efficiency could be demonstrated in a SpCas9 transgenic mouse model, where we observed i. >90% depletion of a target gene product (USP30) at the protein level and ii. population-wide cell transdifferentiation post *Notch2* gene perturbation in distinct epithelial cell types. Notably, after repeat dosing, minimal to no associated inflammation was observed in treated animals. In addition, we observed and characterized AAV vector integration events that occurred at the on-target cleavage site with a frequency of ~12% in lung cells, highlighting a complicating factor with AAV-mediated delivery of DNA nucleases. Taken together, we demonstrate a uniquely effective approach for somatic engineering of the murine lung, which will greatly facilitate the modeling of disease and therapeutic intervention.

487 Optimal Ratio for Nuclease and Donor AAV Vectors in a Genome Editing Approach for Ornithine Transcarbamylase Deficiency

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Our group previously described a genome editing approach for the treatment of ornithine transcarbamylase deficiency (OTCD) in rhesus macaques. OTCD causes lethal episodes of hyperammonemia, and we have proposed a treatment approach in which newborns that survive initial hyperammonemic episodes are treated with gene insertion of a functional copy of the OTC gene. Our genome editing approach consists of dual administration of an adeno-associated virus (AAV) vector expressing an ARCUS meganuclease to create a double-stranded break in a safe harbor site within the *PCSK9* locus and a second AAV vector to deliver a human OTC minigene for knock-in at this site. Following highly successful studies in newborn rhesus macaques in which up to 20% of the liver was expressing human OTC, we evaluated the impact of vector ratio for the meganuclease and donor vectors. We performed studies in a mouse OTCD model that was rendered susceptible to the M2PCSK9 meganuclease through germ-line modification of exon 7 of the endogenous *Pcsk9* gene. After injecting the two vectors into newborn mice at a ratio of 1:1 or 1:3 for the meganuclease to mouse OTC donor vectors, we challenged the mice with a high-protein diet to induce lethal hyperammonemia and evaluated the mice for survival, body weight changes, and transgene expression. By day 7 of the high-protein diet challenge, all untreated OTCD mice had to be euthanized due to a body weight loss of >20% (n=5). In comparison, all mice injected with a low vector dose at a ratio of either 1:1 (both vectors administered at a dose of 10^{13} genome copies [GC]/kg) or 1:3 (10^{13} GC/kg of meganuclease vector and 3×10^{13} GC/kg of donor vector) survived the duration of the high-protein diet challenge (10 days, n=8-10). Both treatment groups exhibited reduced weight loss at day 6 of the high-protein diet challenge (89%-91% of baseline) and increased OTC enzyme activity compared with untreated animals (two- and four-fold for the 1:1 and 1:3 meganuclease-to-donor ratio groups, respectively). Increasing the 1:1 ratio dose so that both vectors were administered at a dose of 3×10^{13} GC/kg resulted in slightly reduced survival by day 10 of the high-protein diet challenge (90% survival) with similarly maintained body weights (91% of baseline) and a two-fold increase in OTC enzyme activity compared with untreated controls. In our previous work, we utilized a 1:3 ratio of meganuclease to donor vectors. The work described here supports this 1:3 ratio as the optimal ratio to efficiently knock-in the human OTC minigene and protect against a high-protein diet in a mouse model of OTCD.

488 DNA Double-Strand Break-Free CRISPR Interference Delays Huntington's Disease Progression in Mice

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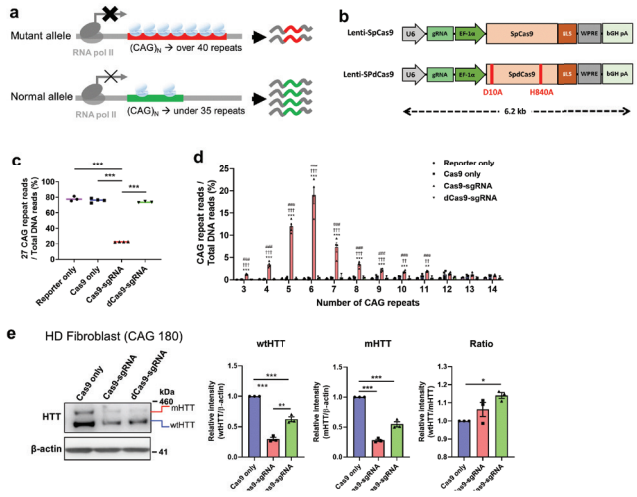
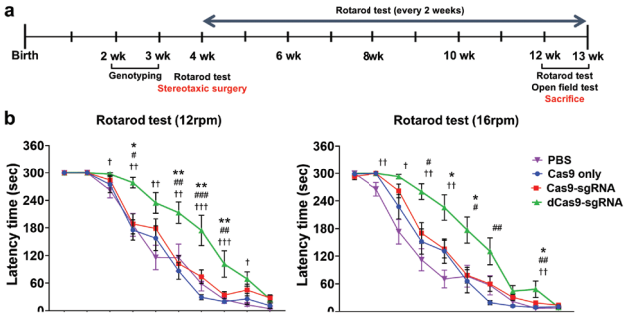
Huntington's disease (HD) is caused by a CAG repeat expansion in the huntingtin (*HTT*) gene. CRISPR-Cas9 nuclease causes double-strand breaks (DSBs) in the targeted DNA that induces toxicity, whereas CRISPR interference (CRISPRi) using dead Cas9 (dCas9) suppresses the target gene expression without DSBs. Delivery of dCas9-sgRNA targeting CAG repeat region did not damage the targeted DNA in HEK293T cells containing CAG repeats. When this study investigated whether CRISPRi can suppress mutant HTT (*mHTT*), CRISPRi resulted in reduced expression of mHTT with relative preservation of the wild-type HTT in human HD fibroblasts.



Although both dCas9 and Cas9 treatments reduced mHTT by sgRNA targeting the CAG repeat region, CRISPRi delayed behavioral deterioration and protected striatal neurons against cell death in HD mice.



Collectively, CRISPRi can delay disease progression by suppressing *mHtt,* suggesting DNA DSB-free CRISPRi is a potential therapy for HD that can compensate for the shortcoming of CRISPR-Cas9 nuclease.This study was supported by a grant from the Korean Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI21C1314, HI22C1588 to S.-R.C.) and the Korean Fund for Regenerative Medicine(KFRM) grant funded by the Korea government (the Ministry of Science and ICT, the Ministry of Health & Welfare) (grant no. 21A0202L1, 21C0715L1 to S.-R.C.).

489 Analysis of Lentiviral Vector-Transduced Hepatocytes from *In Vivo* Gene Transfer Studies

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Lentiviral vectors (LVV) are well suited for the in vivo correction of inborn errors of metabolism in which the liver is the main target for the long-term expression of the therapeutic transgene. However, several challenges remain, such as 1) efficient transduction of target cells within the liver and, 2) achieving therapeutic levels of the transgene. Oxford Biomedica has carried out a series of gene transfer studies to resolve these challenges. Self-inactivating (SIN) third generation LVV have been used extensively for the ex vivo generation of CAR-T cells in the immuno-oncology field and in numerous clinical trials to introduce genes into haematopoietic stem cells for the correction of primary immunodeficiencies and haemoglobinopathies. In addition, in vivo delivery of LVV to the eye has been demonstrated to be safe to generate long-term multi-year stable expression of the therapeutic transgene. The systemic delivery of LVV in animal models has been demonstrated to be safe and efficacious but the systemic delivery of LVV is yet to be demonstrated in humans. We and others have observed species-specific differences in transduction of target cells by LVV. To overcome these differences, we used a mouse model in which human hepatocytes are engrafted into immune deficient FRG mice and repopulate the mouse liver and demonstrated that LVV are more efficient in transducing human hepatocytes in vivo compared to murine hepatocytes. Additionally, hepatocyte-specific promoter (HSP)-driven transgene expression was significantly higher in LVV-transduced human hepatocytes than in AAV-transduced human hepatocytes in this FRG model indicating the potential for high level sustained expression from LVV when administered directly in vivo. Site integration analysis of LVV-transduced C57BL/6 mouse livers revealed a polyclonal integration profile as is typical of LVV, demonstrating the safety of in vivo delivery of third generation LVV. Lastly, single nucleus RNA sequencing (snRNAseq) of livers from LVV-treated mice revealed numerous liver-resident cell subsets which are difficult to identify using histological methods, including various hepatocytes subtypes, hepatocyte progenitors, Kupffer cells, and at least two endothelial cell subsets. Mapping of LVV-encoded transgene transcription driven by HSP is ongoing and will help identify which liver-resident cell subsets transcribe the transgene of interest with great accuracy, enabling the evaluation of cell-targeting technologies. These studies underpin the successful development of LVV for in vivo gene therapy applications for the long-term expression of therapeutic transgenes in humans. Oxford Biomedica is developing a 4th generation LVV system which aims to be able to carry larger transgene/s, with higher expression, additional safety features and higher titres than the current 3rd generation LVV in both ex vivo and in vivo gene therapy applications.

490 Engineered Zinc Finger Transcriptional Regulators Specifically Reduce Prion Expression and Extend Survival in an Aggressive Prion Disease Model

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Prion disease is a rapidly progressing, invariably fatal neurodegenerative disorder caused by misfolding of the cellular prion protein, PrP, encoded by the *PRNP* gene. Most cases are sporadic or caused by inherited dominant mutations in *PRNP*. There are currently no approved or clinical-stage disease-modifying therapies for the prevention or treatment of prion disease. *Prnp* null animals are completely resistant to prion inoculation, and lowering endogenous PrP levels by 50% either genetically or with antisense oligonucleotides can approximately double the lifespan of prion-infected mice. Here, we investigated a single-administration epigenetic regulation approach using AAV-delivered zinc finger transcriptional repressors (ZF-Rs) to achieve sustained and widespread reduction of PrP in the brain and rapid pharmacological effect. Engineered ZF-Rs targeting the transcription regulatory elements of either mouse *Prnp* or human *PRNP* were fused to the human KRAB repression domain and screened in mouse Neuro2A or human SK-N-MC cells, respectively. RT-qPCR analysis identified dozens of highly potent ZF-Rs that reduced prion mRNA by 50-99%. Selected ZF-Rs potently reduced prion mRNA levels in cultured primary mouse cortical and human iPSC-derived neurons with no detectable off-target activity. ZF-Rs under the control of the human Synapsin promoter reduced bulk prion protein mRNA and protein levels by >50% across the brain when administrated to adult mice. Single-cell RNAscope analysis revealed potent *Prnp* knockdown in transduced neurons. PrP was also significantly reduced in mouse

cerebrospinal fluid following AAV-ZF-R administration. Top candidate ZF-Rs were then tested in a prion inoculation mouse model. Wildtype mice were intracerebrally injected with the Rocky Mountain Laboratory (RML) strain of prions (PrP^{Sc}), leading to prion disease symptoms and inevitable mortality approximately 160 days post inoculation (dpi). To evaluate the efficacy of our AAV-ZF-R approach at timepoints relevant to human disease, RML-infected mice were treated with a single dose of ZF-Rs at 60 dpi, near the onset of plasma neurofilament light chain (NfL) rise, or at 122 dpi, near the onset of symptoms and body weight loss. While control groups reached terminal endpoint at 160±8 dpi (mean±sd), a majority of AAV-ZF-R treated mice (n=10/19) were alive at 360 dpi, with attendant improvements in body weight and plasma NfL. In total, 5/19 mice treated with PrP-lowering AAV-ZF-Rs survived to the scheduled necropsy date (500 dpi). Notably, RML-induced increases in plasma NfL levels were arrested following ZF-R treatment at both intervention points. Immunohistochemical staining of brain slices from surviving ZF-R-treated animals revealed a striking reduction in PrP pathology compared to untreated mice. These results support the continued development of a genomic medicine using ZF-Rs for the potential treatment of prion disease.

491 Implementation of Oxford Biomedica Technologies to Improve the Safety and Effectiveness of Lentiviral-Based Liver Gene Transfer

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Liver gene therapy with lentiviral vectors (LVs) holds great potential to provide long-term expression of key enzymes to treat metabolic disorders with a single administration. One key aspect for the success of liver gene therapy is the quality and safety of the LVs. Viral genome (vRNA) splicing and transgene protein contamination in the final product could limit the effectiveness of such therapy. Even when transgene expression during vector production is not detrimental to the out-put titre, the protein could end up in the final drug substance, either incorporated into the virion itself or co-purified with it. This is an issue for liver gene therapy based on intravenous administration of LVs because the transgene present in the vector preparation as a result of the production process could elicit an undesired immune reaction. Over the years, Oxford Biomedica (OXB) has developed key technologies that can address this challenge. Initial *in vitro* data showed that transgene expression in LV producing cells (HEK293T) was occurring even when a liver-specific promoter was adopted. Sequencing data suggested this was the result of aberrant vRNA splicing from the native HIV major splice donor (MSD) that bypasses the rev-response element (RRE) and the liver-specific transcription binding sites on the internal promoter. Splicing of the vRNA and transgene expression were greatly reduced using an inactivated MSD HIV backbone, alongside the OXB U1 technology, which rescues the reduction in titre caused by the MSD-mutation. Any residual transgene expression during vector production was completely abolished by implementing the OXB TRiP System™ (doi: 10.1038/ncomms14834) to work with the liver-specific promoter, with no detrimental effect on production titres. These innovative LV technologies, including the inactivated MSD, U1 technology™ and TRiP System™, were validated in target cells *in vitro*. The results showed that integration efficiency and expression levels were comparable to the original vector, with no vRNA splicing or transgene expression during vector production. These data show how the implementation of OXB innovative technologies can improve the quality and safety of lentiviral vectors for the in vivo delivery of therapeutic transgenes to the liver.

492 Tissue-Specific Gene Editing or Gene Expression Modulation Platform for Safer *In Vivo* Therapeutics

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Precision and safety remain critical concerns for in vivo gene editing-based therapeutics. It is critical for therapeutic development to ensure editing in the target site in the target tissue type with no unintended edits in the genome, and in off-tissues. Recent FDA clinical holds on in vivo gene editing based therapeutics cite the challenges of off-tissue editing, germline editing amongst others. Hundred percent targeted delivery is still an ongoing challenge that is to be solved for. Meanwhile, current gene editing drug designs are incorporating cell/tissue specific promoters to achieve cell specific editing. These come with their own set of challenges such as constantly activated Cas (or other editor) enzymes that cause off targets over the life of patients. Helex's proprietary technology leverages the differential spatial organisations and epigenetic imprints of the genome in various cell/tissue types to introduce precision to editing and prevent off-tissue editing. Helex's Hele-GUIDE platform enables "Double Lock Safety" system where targeted vector delivery (first lock) is supported by tissue-specific Helex gRNA(s) (second lock) to prevent editing in off-tissues despite unwanted vector biodistribution. The AI driven Hele-GUIDE platform identifies unique sequences within the gene or in the gene context (e.g regulators) to edit or modulate the target genes in a tissue-specific manner. The target sequences on the gene are uniquely accessible only in the target tissues compared to off-tissues using a 3D spatial mapping of the chromatin and other epigenetic imprints that favor restricted editing in target tissues. The Hele-GUIDE platform then designs the most suitable gRNAs for the select unique targets considering good on target activity with minimal off-target profiles in both target tissues and off-tissues. Figure1: Hele-GUIDE driven identification of unique hotspots on the genome for cell/ tissue specific editing

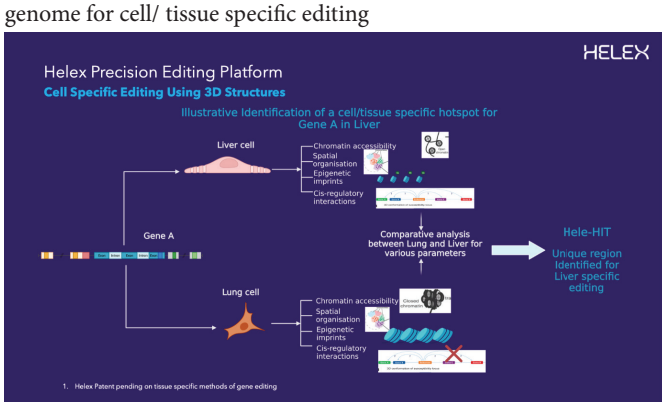
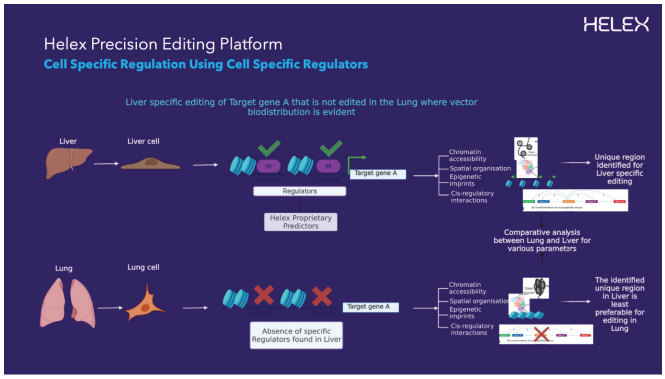


Figure 2: Hele-GUIDE driven identification of unique cell/ tissue specific regulators for specific target gene expression modulation



We have experimentally validated Hele-GUIDE predicted tissue specific targets and their constituent gRNAs for two therapeutically relevant targets- TTR and ANGPTL3. We achieved targeted knock down of the target genes in liver cells (TTR ~50%, ANGPTL3 ~35%) with no editing of target genes in lung, colon cells when all cell types were transfected with the same RNP (gRNA and Cas9) complex via lipofectamine. We have been able to achieve liver specific editing in mice models when the tissue specific RNP complexes were systemically administered via lipid nanoparticles as mode of delivery with biodistribution to liver, spleen, lung, colon and other tissues. Hele-GUIDEs proprietary library comprises of 500+ tissue specific targets spanning over 200 therapeutic indications across the liver and retina that can be leveraged for in vivo gene editing/ gene modulation therapeutics. These results hold promise and great scope for the use of cell specific 3D spatial organization of the genome and epigenetic parameters for gene editing drug design to develop more effective, safer and reliable therapeutics.

493 Precise Genome Editing to Correct MECP2 Mutations in Rett Syndrome

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Rett Syndrome (RTT) is a neurodevelopmental disorder that affects brain development and leads to a progressive loss of movement and intellectual ability. It is caused by mutations in the X-linked MECP2 gene, resulting in a deficiency of the functional MECP2 protein in neurons. MeCP2 is a methyl CpG-binding nuclear protein that acts as a methylation reader which regulates expression of thousands of genes. RTT is almost exclusively observed in heterozygous females, while males with hemizygous MECP2 mutations rarely survive. MeCP2 is universally expressed, but the highest expression levels are observed in neurons. Over 300 distinct MECP2 mutations have been reported in RTT patients, with ~90% located in Exons 3 and 4. Gene therapy of RTT has proven challenging due to a stringent requirement for physiologic regulation of expression, with either too much or too little being toxic. Here we evaluated the possibility of using a genome editing strategy to revert MECP2 mutations back to the wild-type sequence, thus preserving all gene regulatory elements and maintaining physiologic expression levels. The stem cell-derived adeno-associated virus, AAVHSC, have been shown to mediate high-fidelity, precise homologous recombination in the absence of exogenous nucleases. We designed AAVHSC genome editing vectors to correct mutations in Exons 3 and 4 of the MECP2 gene and to insert a promoterless Venus open reading frame (ORF) immediately downstream of the coding region of Exon 4. The MECP2 editing vector was packaged in AAVHSC7 and was used to transduce a panel of RTT patient derived MECP2 mutant cells. Successful editing of the MECP2 gene resulted in the insertion of a promoterless Venus ORF downstream of Exon 4, with expression being driven by the endogenous chromosomal MECP2 promoter. Flow cytometric analysis of AAVHSC-transduced cells revealed specific Venus expression, indicative of successful editing. Editing efficiencies based on Venus expression ranged from 10-20% in a panel of RTT patient-derived primary fibroblasts bearing the R282X, R106W and r.378_384delTCCCCAG MECP2 mutations. For immortalized patient-derived B lymphoblastoid cells, up to 10% of cells were found to be specifically edited. Editing was confirmed at the sequence level, following targeted integration analyses employing insert-specific and chromosome-specific primers. Sanger sequencing of amplicons confirmed that the MECP2 gene was precisely edited. No ITR sequences or insertion / deletion mutations were detected in the edited genomes. Sequence analyses revealed correction of the MECP2 S134C mutation in the genome of edited S134C male RTT cells, which only contain a single mutant X chromosome. Our results clearly demonstrated the capacity of AAVHSC to successfully and accurately correct MECP2 mutations. Editing of the MECP2 gene in female cells, which have both a wild type and a mutant copy of the gene, was confirmed by linkage to unique single nucleotide polymorphisms (SNP) identified in proximity to the edited locus. SNP analyses also enabled mapping of homologous recombination-related crossover events in edited genomes. Interestingly, a higher frequency of recombination was observed closer to the center of the homology region as compared with the periphery. Protein analyses is currently underway to evaluate whether correction of MECP2 mutations restores wild type MeCP2 protein expression. In vivo genome editing studies evaluating correction of a murine Mecp2 mutation is also underway in a mouse model of RTT. In conclusion, we have demonstrated successful correction of mutations in the MECP2 gene associated with RTT using the precise and nuclease-free homologous recombination based AAVHSC genome editing platform.

494 CRISPR/Cas9 Mediated Specific Ablation of *Vegfa* in Retinal Pigment Epithelium Efficiently Regresses Choroidal Neovascularization

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PURPOSE: The CRISPR/Cas9 system easily edits target genes in various organisms and is used to treat human diseases. In most

therapeutic CRISPR studies, ubiquitously expressed promoters, such as CMV, CAG, and EF1α, are used; however, gene editing is sometimes necessary only in specific cell types relevant to the disease. Therefore, we aimed to develop a retinal pigment epithelium (RPE)-specific CRISPR/Cas9 system. **METHODS:** We developed a CRISPR/Cas9 system that operates only in retinal pigment epithelium (RPE) by expressing Cas9 under the RPE-specific vitelliform macular dystrophy 2 promoter (pVMD2). This RPE-specific CRISPR/pVMD2-Cas9 system was tested in human retinal organoid and mouse model. **RESULTS:** We confirmed that this system works specifically in the RPE of human retinal organoids and mouse retina. In addition, the RPE-specific *Vegfa* ablation using the novel CRISPR-pVMD2-Cas9 system caused regression of choroidal neovascularization (CNV) without unwanted knock-out in the neural retina in laser-induced CNV mice, which is a widely used animal model of neovascular age-related macular degeneration. RPE-specific *Vegfa* knock-out (KO) and ubiquitous *Vegfa* KO were comparable in the efficient regression of CNV. **CONCLUSIONS:** The promoter substituted, cell type-specific CRISPR/Cas9 systems can be used in specific 'target cell' therapy, which edits genes while reducing unwanted off-'target cell' effects.

495 An Engineered xCas12i with High Activity, High Specificity and Broad PAM Range

Hainan Zhang[1], Xiangfeng Kong[1], Mingxing Xue[1], Jing Hu[1], Zikang Wang[1], Yinghui Wei[1], Guoling Li[1], Dong Yang[1], Meiling Zhang[2], Yingsi Zhou[1], Hui Yang[1,3]

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Background: Cas12i is characterized by the capability of autonomously processing precursor crRNA to form short mature crRNA and mediates cleavage of dsDNA with a single RuvC domain. Thus, Cas12i is an ideal gene-editing delivery tool via a single AAV. Although Cas12i enables multiplex high-fidelity genome-editing, the natural variants of Cas12i (Cas12i1 and Cas12i2) have shown low editing efficiency thus limiting their utility for therapeutic gene-editing. Here we report the development and usage of a bioinformatic pipeline to annotate Cas12i proteins, CRISPR arrays, and predicted PAM preferences. **Methods**: Fluorescent reporter system was used to assess the activity of the Cas12i proteins by fluorescence intensity. Then, we performed an arginine scanning mutagenesis in the PI, REC-I, and RuvC-II domains of newly discovered Cas12i proteins. To assess the feasibility of engineered Cas12i variants or base-editor for gene-editing *in vivo*, we delivered LNP-packaged mRNA and crRNA to the liver of C57 mice by tail intravenous injection. Finally, we explored the efficiency of engineered Cas12i variant-mediated editing via a single AAV vector in a humanized Duchenne muscular dystrophy (DMD) mouse model, in which mouse exon 51 was replaced by human exon 51 followed by deletion of mouse exon 52. **Results**: We discovered 10 new CRISPR/Cas12i systems, including xCas12i, showing higher fluorescence activity than SpCas9 and LbCas12a. Engineered xCas12i variant, hfCas12Max, exhibits higher efficiency than several current Cas proteins, broadens PAM (5'-TN or 5'-TNN PAM), and exhibits high specificity in mammalian cells (Fig. 1A-C). Additionally, engineered dxCas12i-based editors exhibit high base-editing activity. Through delivering hfCas12Max RNP targeting *TRAC* in primary human T cells, crRNA-2 and crRNA-3 generated ~90% editing and reduced to 2-3% level of TRAC expression along with ~80% viability in CD3+ T cells (Fig. 1D). By targeting the exon 3 of the transthyretin (*Ttr*) gene by gene and base-editing in mice, we detected ~70%, equivalent to saturation, editing efficiencies in murine liver using targeted deep sequence analysis (Fig. 1E). Furthermore, TadA8e-dxCas12i-v4.3 achieved ~25% A-to-G editing efficiency of A13 in the *Ttr* locus in murine liver through LNP delivery. After systemic delivery of a single all-in-one AAV vector containing hfCas12Max and a crRNA targeting the splice donor site of human exon 51 to the DMD mouse model, the dystrophin expression was efficiently restored as well as histopathology and grip strength were ameliorated. In further exploration to neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), hfCas12Max-mediated knockdown efficiency of toxic proteins exceeded 80%. **Conclusions**: These results indicate that hfCas12Max mediates robust editing activity and high specificity. hfCas12Max is a versatile platform for genome- or base-editing in mammalian cells or mice and could be useful in the future for *in-/ex-vivo* therapeutic applications, showing significant potential for disease modeling and therapies.

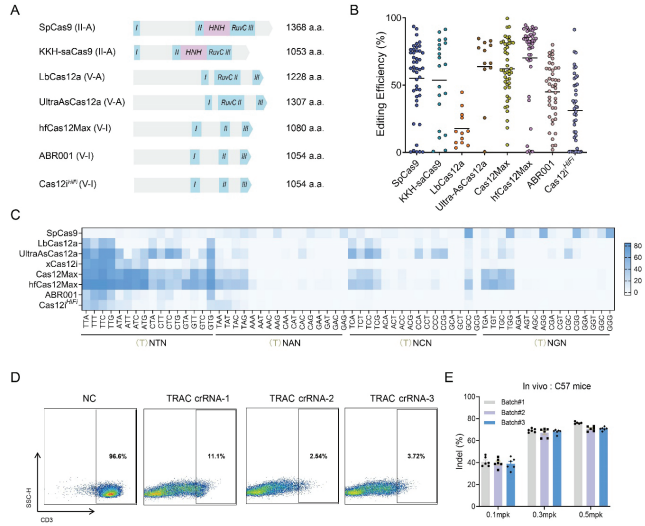

Figure 1. hfCas12Max, an engineered natural variant xCas12i, mediated high-efficient genome editing in mammalian cells, *ex vivo* and *in vivo*. A, Domains and sizes of several CRISPR Cas proteins. **B**, Comparison of indel activity from Cas12Max, hfCas12Max, LbCas12a, Ultra AsCas12a, SpCas9, KKH-saCas9, ABR001 and Cas12i^{HiFi} at *TTR* locus. hfCas12Max showed higher gene-editing efficiency than other Cas proteins. Each dot represents one of three repeats of single target site. **C**, hfCas12Max exhibited a broader PAM recognition profile than other Cas proteins, including 5'-TN and 5'-TNN PAMs. **D**, Representative flow cytometric analysis of edited CD3+ T cell 5 days after RNP delivery. NC represents blank control, untreated with RNP. **E**, Indel rates of LNP packaging with hfCas12Max mRNA and targeted *Ttr* crRNA at three doses (0.1, 0.3 and 0.5 mpk) in C57 mice (n=6).

496 Adenine Base Editing of Galactosylceramidase Gene in a Mouse Model of Krabbe's Disease

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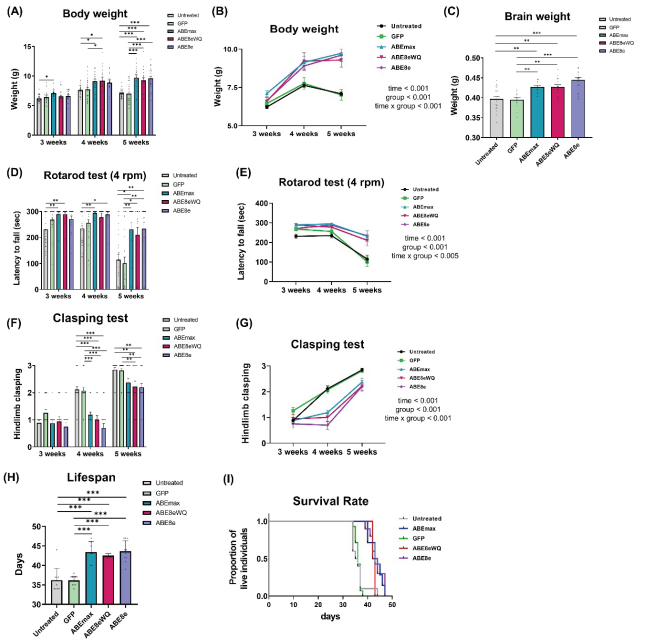
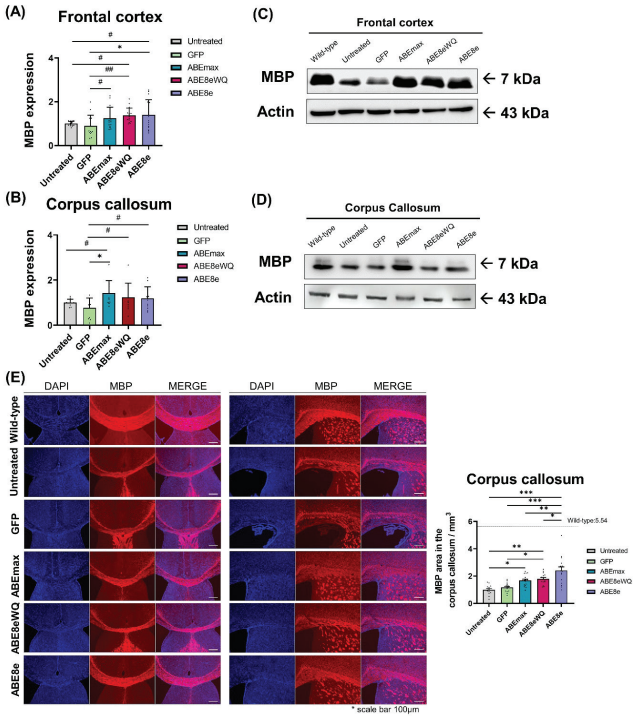
Cure for globoid cell leukodystrophy, Krabbe disease (KD) has long been in demand. A base editing has emerged as a promising treatment. Unlike CRISPR-Cas9 repair system, adenine base editor (ABE) can correct target sequences without DNA templates which prevents double-stranded DNA breaks. ABE has emerged as an exclusive therapeutic candidate among base editing systems with the possibility to correct a mutant gene for the recovery of the lost enzyme function. When the ABE gene was separated into two adeno-associated virus (AAV) vectors, in vitro transfection experiment of the ABE gene showed no significant difference between the unseparated and split genes. High-throughput sequencing showed 37 and 56% of the base editing efficiency. In qRT-PCR, mRNA expression levels of split-ABE pairs were largely shown in the corpus callosum and frontal cortex where also showed 0.5 and 3% of the base editing efficiency. The ABE treatments significantly ameliorated neurobehavioral functions in rotarod and clasping tests as well as body weight. Finally, brain weight and life span were significantly alleviated compared to untreated and GFP groups.



The brain of ABE-treated mice exhibited myelin recovery in qRT-PCR, Western blot, immunohistochemistry and transmission electron microscopy in addition to reduced globoid cell pathology in Luxol fast blue/periodic acid-Schiff staining and preserved axonal integrity in diffusion tensor imaging of the brain MRI.



These data indicated the novelty of a base editing technology using ABEs as a fundamental treatment option for KD, potentially extending a promising challenge for various genetic diseases.This study was supported by a grant from the Korean Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI21C1314, HI22C1588 to S.-R.C.) and the Korean Fund for Regenerative Medicine(KFRM) grant funded by the Korea government (the Ministry of Science and ICT, the Ministry of Health & Welfare) (grant no. 21A0202L1, 21C0715L1 to S.-R.C.).

497 Molecular Characterization of CRISPR-Based Targeted Gene Insertion of *Factor 9* as a Potential Treatment for Hemophilia B

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CRISPR/Cas9-mediated genome editing is an emerging approach to treat human diseases by addressing their underlying genetic cause. We have developed CRISPR-based therapeutic platforms to either reduce or restore expression of genes involved in human disease. One such platform is a CRISPR-based gene insertion approach, which involves the targeted insertion of a promoterless expression cassette into the *Albumin* locus of liver hepatocytes using a lipid nanoparticle (LNP) and adeno-associated virus (AAV) delivery system to allow robust and durable therapeutic protein expression from the liver. We have applied this approach to hemophilia B, an X-linked genetic

disorder characterized by reduced coagulation Factor IX (FIX) activity. In adult hemophilia B mice, we have established that liver insertion-derived FIX protein is functionally indistinguishable from purified human (h) FIX, and treatment with our development candidate rescues hemostasis in hemophilia B mice. To further characterize our insertion platform, we have developed a suite of molecular biology assays that quantitatively characterize *in vivo* DNA insertion and subsequent RNA transcription. We applied our suite of assays to characterize the insertion outcomes in adult hemophilia B mice that received hFIX-AAV and Cas9-guide RNA (gRNA) LNP. At the DNA level, AAV-LNP treatment resulted in dose-dependent increases in insertion events (quantified by droplet digital PCR) and indels at the *Albumin* locus (quantified by targeted amplicon sequencing). At the RNA level, AAV-LNP treatment resulted in dose-dependent increases of ALB-hFIX fusion transcripts (quantified by reverse transcriptase quantitative PCR [RT-qPCR] and by whole transcriptome RNA sequencing). In addition, at the RNA level, AAV-LNP treatment resulted in dose-dependent increases in the percentage of cells expressing ALB-hFIX fusion transcripts (measured by BaseScope *in situ* hybridization assays). By characterizing the effects of our AAV-LNP treatment at the DNA, RNA, and protein levels, we can correlate the extent of gene insertion to FIX expression (RNA and protein). This will aid us in using this model for dose selection and gain mechanistic understanding of our platform. Our targeted gene insertion approach has the potential to become a durable solution to several monogenic diseases.

498 Targeted Delivery of Genome Editors Complexed with Guide RNA Using ARMMs for Non-Viral *In Vivo* Delivery

Qiyu Wang, Shu-Lin Liu, Ryan Kleeck, Chris Chatzinakos, Komal Vyas, Chou-Wei Chang, Wendy Zhao, Carson Semple, Lucy Sun, Pearl Moharil, Zahra Baghbantaraghdari, Jennifer Badji, Kristin Luther, Adam Francoeur, Joseph F. Nabhan

Vesigen Therapeutics, Cambridge, MA

In vivo gene editing therapies offer the transformative potential to cure, ameliorate, or prevent an array of diseases. However, broader therapeutic applications, beyond targeting hepatocytes or *ex vivo* gene editing, have been limited by the inability to efficiently and safely deliver gene editing technologies to disease-affected tissues and cell types. We have engineered the human vesicles ARMMs (ARrestin-domain 1 Mediated Microvesicles) as a safe, targeted, and re-dosable therapeutic platform for the delivery of CRISPR/Cas9 gene editing complexes *in vivo*. We utilize the protein ARRDC1, which is the principal driver of ARMM formation, as a recruitment handle to actively load engineered vesicles. That CRISPR/Cas9 ribonucleoprotein-loaded ARMMs are engineered versions of naturally existing vesicles enables this delivery system to overcome various limitations of existing delivery technologies, such as toxicity, low specificity, instability, and immunogenicity. We have developed a scalable cell-based production system to readily load the CRISPR/Cas9 protein complexed to guide RNA (gRNA) into ARMMs and achieve high loading efficiencies, unparalleled by other platforms. Functional delivery of Cas9/gRNA complexes by ARMMs was first demonstrated *in vitro* with multiple gRNA sequences targeting several genomic loci in a range of human and murine cell types, including primary and post-mitotic cells. *In vivo* administration of ARMMs yielded 60-70% editing after a single dose. These results highlight the therapeutic potential of ARMMs-based delivery of CRISPR/Cas9 in disease modification.

499 CRISPR PERSIST-On Enables Heritable and Fine-Tunable Human Gene Activation

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Current technologies for upregulation of endogenous genes use targeted artificial transcriptional activators but stable gene activation requires persistent expression of these synthetic factors. Although general "hit-and-run" strategies exist for inducing long-term silencing of endogenous genes using targeted artificial transcriptional repressors, to our knowledge no equivalent approach for gene activation has been described to date. To address this, here we show stable gene activation can be achieved using a strategy we call Precision Editing of Regulatory Sequences to Induce Stable Transcription-On (**PERSIST-On**). This approach leverages the activities of endogenous transcription factors (**EndoTF**s) that are normally expressed in human cells and can be used to induce stable long-term gene activation, which we show is durable for at least five months. We also show how PERSIST-On can be modulated in two different ways to fine tune the magnitude of gene activation observed. Taken together, our results delineate a generalizable platform for inducing heritable and fine-tunable gene activation in a hit-and-run fashion, thereby enabling a wide range of research and therapeutic applications that require stable long-term upregulation of a target gene.

500 Expansion of CD33-Edited CD34$^+$ Cells by Gemtuzumab Ozogamicin for *In Vivo* HSC Gene Therapy of Hemoglobinopathies

Aphrodite Georgakopoulou[1], Sucheol Gil[1], Hans-Peter Kiem[2], Andre Lieber[1], Chang Li[1]

[1]University of Washington, Seattle, WA,[2]Fred Hutch, Seattle, WA

We have previously developed a new *in vivo* HSC gene therapy approach that involves HSCs mobilization and intravenous injection of helper-dependent adenovirus (HDAd5/35++) vectors that target receptors present on primitive HSCs. HSCs transduced in the periphery return to the bone marrow and spleen and persist there long-term. The approach has recently been used for *in vivo* editing of HSCs to reactive γ-globin or correct the Sickle Cell Disease mutation. In order to achieve therapeutically relevant editing levels, the current approach required several rounds of *in vivo* selection with low-dose chemotherapy drugs. Roland Walter and Hans-Peter Kiem developed a new system that could potentially be used for *in vivo* selection of edited HSCs and expansion of edited erythroid cells. It consists of a CRISPR-Cas9 to knock-out the exon 2 of CD33 gene in HSPCs and gemtuzumab ozogamicin (GO),

an immunotoxin that targets non-transduced, CD33$^+$ HSPCs. Notably, CD33 is dispensable for normal hematopoiesis. Here we developed a multiplex adenine base editing approach, by using a HDAd5/35++ vector to simultaneously: *i)* mutate the splice acceptor site to eliminate the second exon of CD33 (which encodes for the GO target epitope) and *ii)* inactivate a BCL11a repressor site within the HBG1/2 promoters to reactivate γ-globin (HDAd-ABE8e-sgHBG-sgCD33). Transduction of a CD33$^+$ myeloid cell line (ML-1 cells) followed by GO selection resulted in nearly 100% editing of both target sites and >98% CD33$^{\Delta E2}$ cells at day 14, indicating that editing/selection system worked efficiently. More than 80% of CD34$^+$ cells express medium or high levels of CD33, a marker for common myeloid/progenitor cells. We therefore hypothesized that knockout introduction of CD33$^{\Delta E2}$ in these cells by the base editor vector would allow for GO-mediated expansion of edited erythroid cells with reactivated γ-globin. In a first study, CD34$^+$ cells were transduced with the HDAd-ABE8e-sgHBG-sgCD33 vector and cultured for 7 days in low cytokine medium, supplemented with the small molecules SR1 and Ly, which are known to preserve the stemness of CD34$^+$ cells in culture (as reflected by the percentage of CD34$^+$/CD38$^-$/CD90$^+$ cells), thus allowing for target site editing to occur before the start of GO selection. On day 7, cells were subjected to increasing concentrations of GO and then incubated in erythroid differentiation medium for 18 days, or myeloid differentiation medium for 14 days. Editing of the target CD33 and HBG1/2 sites was measured before GO treatment and after GO treatment/*in vitro* differentiation. Preselection editing frequency at day 7 after HDAd transduction were ~3% for the CD33 and HBG1/2 sites, respectively. The relatively low editing rate could be HSC donor related. *In vitro* selection of HDAd-ABE8e-sgHBG-sgCD33-transduced CD34$^+$ cells with GO resulted in 10% and 30% edited CD33 and HBG1/2 alleles, respectively, at the end of erythroid differentiation. This led to an increase of HbF expressing erythroid cells (HDAd transduced: 80% vs non-transduced: 43% HbF$^+$ cells). (Notably, the erythroid differentiation medium actives γ-globin expression to some degree.) A similar increase in editing rates after GO treatment was observed after incubation in myeloid differentiation medium. After 14 days of myeloid differentiation, ~10% of cells were CD33$^{\Delta E2}$, compared to ~2% of non-transduced cells that underwent GO treatment and myeloid differentiation, indicating the expansion of myeloid cells where the base editor had knocked-out the CD33 exon 2. Optimization of the editing efficacy and GO concentrations for selection of double edited CD34$^+$ cells *in vitro* (with additional CD34$^+$ cell donors) and *in vivo,* in humanized mice, are ongoing. Overall, these data indicate that CD33-based selection of edited HSPCs could improve our *in vivo* HSC gene therapy approach.

501 *In Vivo* Transduction of Mobilized Human CD34$^+$ Cells in a Humanized Mouse Model with HDAd5/35++ and HDAd6/35++ Vectors

Aphrodite Georgakopoulou[1], Hongjie Wang[1], Hans-Peter Kiem[2], Chang Li[1], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Fred Hutch, Seattle, WA

We have previously developed a minimally invasive and readily translatable approach for *in vivo* HSC gene delivery without leukapheresis, myeloablation, and HSC transplantation. It involves injections of G-CSF/AMD3100 to mobilize HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of integrating, helper-dependent adenovirus vector systems based on HDAd5/35++ and them, recently developed, HDAd6/35++ platforms. Both vector platforms target CD46, a receptor that is expressed on primitive HSCs. Transgene integration is achieved using a hyperactive *Sleeping Beauty* transposase (SB100x). Current vectors also contain an mgmt^{P140K} expression cassette which allows for *in vivo* selection of transduced HSCs with low-dose O^{6}BG/BCNU. The efficacy and safety of the system has been shown in studies with human CD46-transgenic mice and rhesus macaques. The generation of a humanized mouse model and successful *in vivo* transduction of CD34$^+$ cells are important for the development of new *in vivo* selection approaches that cannot be tested in human CD46tg mice, such as the use of immunotoxins specifically targeting human HSCs receptors or expansion of HSCs using epitope engineering of receptors present on human HSCs. We therefore generated a xenografted mouse model, by transplanting CD34$^+$ cells from healthy donors into NBSGW mice to establish human hemopoiesis. Six weeks post transplantation, CD34$^+$ cells from the chimeric murine bone marrow were successfully mobilized to the periphery by administration of G-CSF (250μg/kg for 6 days) and AMD3100 (5mg/kg for 4 days). The mobilization resulted in 37,448±7,818 circulating human CD34$^+$ cells forty minutes after the last AMD3100 injection. At this time point, mice were intravenously injected with GFP-expressing HDAd5/35++ or HDAd6/35++ vectors. Three days post transduction, no differences between HDAd5/35++-GFP and HDAd6/35++-transduced cohorts were observed in multi-lineage engraftment rates of human cells in bone marrow and spleen. Importantly, the *in vivo* transduction with both HDAd vector resulted in (on average) 10% and 2% GFP marking of CD34$^+$ cells in the bone marrow and spleen, respectively, at day 3 after HDAd injection. GFP marking in non-mobilized animals was not above background. This study shows that *i)* CD34$^+$ cells can be efficiently mobilized in humanized mice, *ii)* mobilized CD34$^+$ cells can be transduced with intravenously injected HDAd vectors targeting CD46, and *iii)* that transduced cells return to the bone marrow and spleen. A 10% marking rate in bone marrow CD34$^+$ cells is a solid basis for testing new in vivo HSC selection/expansion approaches in humanized mice.

502 Engraftment of Hepatocytes Gene-Edited *Ex Vivo* for the Treatment of Inherited Metabolic Liver Disease

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Background Inherited metabolic diseases (IMD) of the liver occur once in 800 live births and are typically caused by autosomal recessive single-gene mutations resulting in organ intoxication and premature death if untreated. An example of an IMD of the liver is hereditary tyrosinemia type I (HT-1) caused by non-functional mutations in the gene encoding the enzyme fumarylacetoacetate hydrolase (FAH). Liver transplantation represents the only curative therapy for IMDs of the liver; however, the limitations, including severe organ shortages, the life-long requirement for immunosuppressant drugs, and the high risk of mortality, highlight the urgent need for novel therapies. A novel

therapeutic approach consists of ex vivo gene editing using CRISPR-Cas9 nucleases to disrupt the gene encoding 4-hydroxyphenylpyruvate dioxygenase (HPD), an enzyme upstream in the metabolic pathway, in hepatocytes isolated from the patient's resected liver. The gene-edited hepatocytes would be subsequently transplanted into the liver to correct the disease phenotype. Although a promising tool for treating IMDs of the liver, the major challenge of CRISPR-Cas9 is its delivery into hepatocytes. Adeno-associated virus vectors (AAV) are the standard delivery method for introducing CRISPR-Cas reagents, but AAVs are associated with severe safety and efficacy concerns having the potential to hamper the advancement of gene editing therapies in the clinic. Here, we investigate the delivery of CRISPR-Cas9 into hepatocytes using electroporation and evaluate the engraftment of edited hepatocytes in the liver.**Methods** After perfusing the liver using a 3-step enzymatic degradation method to isolate hepatocytes from wild-type C57BL/6 or Fah -/- mice, we used electroporation to deliver Hpd-aiming CRISPR-Cas9 RNPs into hepatocytes. Gene-edited hepatocytes were injected via the spleen into Fah-/- recipient mice. NTBC was cycled on and off to promote engraftment.**Results** In preliminary studies, we optimized the delivery of CRISPR-Cas9 nucleases targeting the Hpd and observed up to 70% on-target activity with 100% survival and complete liver repopulation following splenic injection. We then investigated the effects of CRISPR editing on engraftment as well as the effect of using a cytokine media cocktail after electroporation. In mice transplanted with gene-edited wild-type hepatocytes incubated in cytokine media, we observed up to 47% engraftment, while no cytokine group showed no engraftment (<2%). All the transplant mice were rescued from HT-1. Next, we used CRISPR-Cas9 to disrupt Hpd ex vivo to reprogram the metabolic pathway. We observed an average of 35% engraftment for Hpd-Cas9 RNP and 28% for Hpd-Cas9 mRNA. Hpd protein concentration decreased significantly by 3-fold in both treatment groups. When the viable hepatocyte number was increased, the engraftment level was further boosted to up to 75%. Moreover, we showed that the Hpd knock-down using CRISPR-Cas9 protects Fah-/- mice from liver failure.**Conclusions** The cytokine media is found to be critical for the engraftment capacities of primary hepatocytes after electroporation. Our results indicate that hepatocytes edited using CRISPR-Cas9 delivered by electroporation have the capacity to engraft in the liver and correct acute liver failure a HT-1 mouse model.

503 Glycosaminoglycan-Binding Enhanced Transduction (GET) Peptide Nanoparticles for Cystic Fibrosis Gene Therapy

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Cystic fibrosis (CF), one of the most common genetic diseases, is resulted from a mutation in the gene encoding the CF transmembrane conductance regulator protein. It causes lung epithelial ionic dysregulation and ultimately lung function decline. Given the monogenic nature of CF and the direct accessibility of the lung airways, inhaled gene therapy has been considered attractive and straightforward. However, early clinical trials failed to show a meaningful outcome due to the limited ability of gene vectors to overcome biological barriers, including airway mucus, and to achieve therapeutically relevant gene transfer efficacy. We previously demonstrated that arginine-rich GET peptide (FLR) nanoparticles (NPs) enhanced intracellular delivery of various therapeutic cargos, including nucleic acids. We then showed that surface shielding with polyethylene glycol (PEG) facilitated the penetration of FLR NPs through human lung airway mucus and enhanced transfection efficiency in mouse lungs following intratracheal administration. To further improve the performance of our NPs, we have engineered a mucus-penetrating formulation that includes polyhistidine (FLH) to enhance endosome escape via the proton sponge effect (PEG-FLR/FLH) and compared with previous formulations (FLR and PEG-FLR). All three formulations exhibited similar particle hydrodynamic diameters of ~50 nm (**A**) and the positive surface charge inherent to FLR was neutralized by PEGylation (**B**). The particle morphology and diameter were also monitored by transmission electron microscopy (**C**). We next conducted in vitro studies where we found that PEG-FLR/FLH, despite the surface shielding of the positive charge that promotes cellular uptake, provided greater transfection efficiency compared to branched PEI (bPEI) without significant cytotoxicity (**D**). Our multiple particle tracking analysis revealed that both PEGylated formulations, due to the ability to minimize adhesive interactions with mucus constituents, exhibited markedly greater mobility in CF sputum compared to FLR NPs and polystyrene beads (**E-F**), in agreement with our previous reports. Finally, we conducted an in vivo study where we treated healthy inbred mice with either PEG-FLR or PEG-FLR/FLH carrying luciferase-expressing plasmids via intratracheal administration and evaluated transfection efficiency. The PEG-FLR/FLH provided significantly greater transgene expression compared to PEG-FLR, most likely due to the ability of FLH to promote endosome escape. Encouraged by these outcomes, we are currently pursuing further mechanistic studies and testing our NPs using a mouse model with thickened mucus barrier as shown in the lungs of patients with CF.

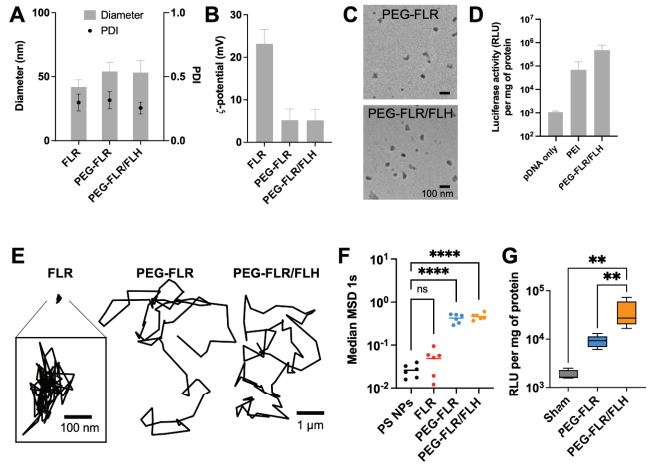

Figure 1. PEG-FLR/FLH provides efficient mucus penetration and lung transfection. Different non-PEGylated or PEGylated peptide-based gene delivery nanoparticles (NPs) were prepared and compared. (**A**) Hydrodynamic diameter, (**B**) zeta-potential, and (**C**) transmission electron micrographs of NPs. (**D**) In vitro transfection efficiency of NPs. (**E, F**) Multiple particle tracking analysis show the ability of NPs to penetrate CF sputum ex vivo. (**E**) Representative trajectories of different NPs moving within CF sputum samples and (**F**) Quantitative analysis. Median mean square displacement (MSD) values

are directly proportional to the particle diffusion rates. (**G**) In vivo luciferase transgene expression in the lung mediated by intratracheal administered NPs.

504 Development and Characterization of New Reporter Mouse Models for *In Vivo* Gene Editing

Kathy J. Snow[1], Caleb Heffner[1], Yaned Gaitan[1], Ethan Saville[1], Lawrence Bechtel[1], Tiffany L. Davis[1], Wolfgang Wurst[2], Ralf Kuehn[3], Stephen A. Murray[1], David E. Bergstrom[1]

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With the evolution and expansion of therapeutic CRISPR/Cas9 gene editing technologies, reliable, efficient small animal models are needed to evaluate the function of these new tools *in vivo*. To improve on existing reporters such as Ai9, JAX's Small Animal Testing Center (SCGE Consortium) has developed several new gene editing reporter alleles based on a "traffic light" reporter design. The new reporters can be used to detect a variety of DNA repair outcomes following editing with a single guide, including non-homologous end joining (NHEJ), homology-directed repair (HDR) and base editing. These models include PAM sites for multiple Cas9 variants, and in some cases have been designed to bias repair outcomes to improve the rate of reporter activation following an editing event. Each reporter line has been characterized for both baseline reporter functionality in cultured mouse embryos, and has been validated in for *in vivo* somatic gene editing applications through extensive characterization of a standard panel of adult tissues. Together, these new models provide a valuable resource for improved detection of genome editing events and expands the breadth of nucleases and editing outcomes that can be tested *in vivo*.

505 Base Editing Correction of DMD in Human iPSC-Derived Cardiomyocytes and Dystrophic Mice

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Indiana University School of Medicine, Indianapolis, IN

Duchenne muscular dystrophy (DMD) is a devastating muscular disease that is caused by mutations in the *DMD* gene. Gene editing has shown great promise in correcting the genetic base for DMD. In this study, we used base editing to correct a nonsense mutation or to induce exon skipping in order to rescue dystrophin expression. Firstly, we rationally improved the NG-targeting adenine base editor (iABE-NGA) and demonstrated the high efficiency of precisely editing in adult dystrophic mice. Systemic delivery of AAV9-iABE-NGA resulted in dystrophin restoration and functional improvement. At 10 months after AAV9-iABE-NGA treatment, a near complete rescue of dystrophin was measured in *mdx*^{4cv} mouse hearts with up to 15% rescue in skeletal muscle fibers. The off-target activities remained low, and no obvious toxicity was detected. To broaden the applicability of base editing therapeutic approach for DMD caused by large deletions (~68% of all cases), we further explored the feasibility of base editing to induce exon skipping in DMD cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs). We generated a DMD hiPSC line with a large deletion spanning exons 48 through 54 (ΔE48-54) using CRISPR-Cas9 gene editing. Dystrophin expression was disrupted in DMD hiPSC-derived cardiomyocytes (iCMs) as detected by RT-PCR, Western blot, and immunofluorescence staining. Co-transfection of ABE and a guide RNA (gRNA) targeting the splice acceptor led to efficient conversion of AG to GG (35.9% ± 5.7%) and enabled exon 55 skipping. Complete AG to GG conversion in a single clone restored dystrophin expression (42.5% ± 11% of wild type [WT]) in DMD iCMs. Moreover, we demonstrated the efficiency of inducing exons 6, 7, 8, 43, 44, 46, and 53 skipping in iCMs. These results highlight base editing with iABE-NGA as a promising therapeutic approach for DMD.

506 Epigenetic Editing of the CDKL5 Gene in Mouse and Human Cells

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CDKL5 deficiency disorder (CDD) is an early infantile epilepsy and X-linked intellectual disability caused by de novo mutations in the CDKL5 gene. In female somatic cells, one copy of CDKL5 becomes epigenetically silenced by X chromosome inactivation (XCI). In cells expressing the mutant allele, reactivation of wildtype *CDKL5* could present a paradigm shift in the treatment of X-linked disorders by partially or fully restoring disease-associated phenotypes. In 2021, a landmark paper demonstrated that conditional restoration of *Cdkl5* using a genetic mouse model could reverse functional deficits across multiple domains even in adult mice. These results demonstrate the utility of restoration of *Cdkl5* as a treatment for CDD. We have previously demonstrated that targeted *CDKL5* reactivation is feasible in human neuronal-like cells using a dCas9 epigenetic editor. In addition, we have demonstrated that AAV delivery of such epigenetic editors is possible in the mouse brain. Our studies found that AAV particles containing epigenetic editors both result in significant *Cdkl5* upregulation and targeted removal of methylation from the mouse *Cdkl5* promoter. Our experiments utilize an improved AAV cassette that reduce viral packaging size through the use of a split dCas9, small promoters and a synthetic polyA signal and the use of a sgRNA-tRNA cassette that allows expression from a single U6 promoter. Ongoing studies address rescue of phosphorylation substrates EB2 and MAP1S. In addition, lentiviral delivery of our novel dCas9 editors to patient-derived neuronal stem cells and induced neurons has been used to measure *CDKL5* promoter methylation changes and gene reactivation. In addition, long-term exposure of lentiviral particles has been used to address rescue of the known CDKL5 phosphorylation substrates. This approach holds great promise for those affected by CDD.

507 PAMless Base Editors as a Genetic Treatment for Spinal Muscular Atrophy

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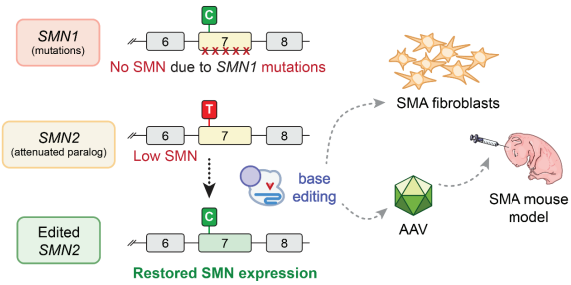
Spinal muscular atrophy (SMA) is a devastating neuromuscular disease caused by mutations in the SMN1 gene. Despite the development of various therapies, outcomes can remain suboptimal in SMA infants and the duration of such therapies are uncertain. SMN2 is a paralogous gene that mainly differs from SMN1 by a C•G-to-T•A transition in exon 7, resulting in the skipping of exon 7 in most SMN2 transcripts and production of only low levels of survival motor neuron (SMN) protein. Genome editing technologies targeted to the SMN2 exon 7 mutation could offer a therapeutic strategy to restore SMN protein expression to normal levels irrespective of the patient SMN1 mutation. Here, we optimized a base editing approach to precisely edit SMN2, reverting the exon 7 mutation via an A•T-to-G•C base edit. We tested a range of different adenosine base editors (ABEs) comprised of engineered TadA domains and CRISPR-Cas9 enzymes, and observed the most specific editing (with minimal bystander editing) when using the PAMless enzyme ABE8e-SpRY. In SMA patient-derived fibroblasts, we observed up to 99% intended editing with concomitant increases in SMN2 exon 7 transcript expression and SMN protein levels. We generated and characterized ABEs fused to high-fidelity Cas9 variants which reduced potential off-target editing in HEK 293Ts, and also observed only very low-level off-target editing in SMA fibroblasts. Delivery of these optimized ABEs in vivo via dual adeno-associated virus (AAV) vectors resulted in precise SMN2 editing in an SMA mouse model. This base editing approach to correct SMN2 should provide a long-lasting genetic treatment for SMA with advantages compared to current nucleic acid, small molecule, or exogenous gene replacement therapies. More broadly, our work highlights the potential of PAMless SpRY base editors to install edits efficiently and safely.



508 Nanopore Sequencing Identifies Miscellaneous Insertion Events after Double-Cut Donor-Mediated *In Vivo* CRISPR Editing

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CRISPR-Cas9-enabled precise insertion of a large transgene in target cells is transformative to in vivo gene therapy of numerous diseases, such as hemophilia A. Expression of B-domain-deleted F8 by hepatocytes has clinically demonstrated to be effective to this hemostasis disorder, while the safety concerns of gene editing still hamper its wide application. Multiple approaches developed before addressed off-target effects and large deletions using Illumina or nanopore sequencing (NP-seq). However, on-target integration of a large fragment has yet to be comprehensively documented, particularly in *in vivo* editing, where template integration is often inefficient. To address this challenge, we developed an approach to characterize the complexity of inserted sequences at a CRISPR-targeted locus. Previously, in a hemophilia A mouse model, we showed that hydrodynamic injection of an F8 double-cut donor and CRISPR plasmids induced integration of BDDF8 at Alb locus at an efficiency of ~1% and restored the F8 to normal levels. This model allowed us to investigate the insertions in the liver. Optimizing long-range PCR allowed us to amplify 5-10 kb flanking the *Alb* target site. Still, NP-seq showed only ~0.05% of reads with donor insertion due to biased amplification of short background sequence. Therefore, we treated DNA with the RNP complex of Cas9-sgAlb and observed a 10-fold enrichment of reads with donor insertion. We designed primers amplifying a ~400-bp background to deplete the unwanted alleles further. Two rounds of 0.4x Magbeads purification of the 1st PCR product led to over 100-fold depletion of short fragments. However, we observed 5-10 times higher read numbers with plasmid backbone (BB; ~2 kb) than the F8 cassette (~6 kb). To attenuate the preferential BB amplification, we treated 1st PCR product with a BB-targeting RNP and observed more balanced events. To enable pooled NP-seq, we used primers with 12-bp indel-correcting barcodes for 2nd PCR. We also developed an algorithm to retrieve data from pooled NP-seq data, with an over 50% recovery and less than 0.1% false discovery rate (FDR), and a pipeline termed GREPore-seq for demultiplexing, quality control, and data visualization. Using the optimized protocol, ~80% of reads contained inserts. As expected, most insertions were BB or F8 in forward or reverse orientation due to intracellular linearization of the double-cut donor. We also observed the insertion of 2 or 3 pieces at the DNA break site, albeit at low frequencies. Among the possible 80 patterns of two and three fragments of donor plasmid integrations, we detected 55. Besides, the insertion of Cas9-sgRNA plasmid fragments was found in ~0.01% of reads. Consistent with previous work, we also found that ~0.01% of inserts originated from mouse chromosomes, and ~0.002% of events might be mediated by LINE-1 reverse transcriptase. Together, our data demonstrate that CRISPR cleavage enables the effective insertion of donor sequences, but a trace amount of genomic fragments and LINE sequences are

incorporated at DSB sites. Reassuringly, no abnormalities in the liver and other organs had been observed in over 100 mice that had been followed up on for 1 year. In conclusion, for the first time, our study unveils the complex CRISPR-mediated on-target insertion outcome after in vivo gene editing therapy. This robust, affordable Insert-seq approach will find its application in AAV gene therapy for identifying the integration spectrum of AAV.

509 Prime Editing Leads to Phenotypic Correction in a Novel Mouse Model of Fanconi Anemia

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Fanconi anemia (FA), resulting from germline mutations in DNA repair genes of the FA/BRCA pathway, is the most prevalent inherited bone marrow failure disorder. The only approved treatment for FA is hematopoietic stem cell (HSC) transplant from a healthy donor, but severe post-transplantation complications are common, and in some cases lethal. To overcome these risks, *in vivo* or *ex vivo* gene editing of autologous HSCs to correct the underlying mutation has been proposed. As the defining DNA repair defect of FA cells precludes homologous recombination-dependent gene editing, we have implemented *in vitro* prime editing in primary HSCs from a novel mouse model with an human FA patient mutation (FancL^{TATdel}).Mice that are homozygous for the FancL^{TATdel} allele display abnormalities consistent with human FA, including reproductive defects, hypersensitivity to the DNA damaging agent mitomycin C, and reduced hematopoietic stem and progenitor (LSK+) cell numbers in older mice. We utilized HOXA9 immortalized myeloid cells derived from FancL^{TATdel} mice, or an EGFP reporter mouse, to optimize conditions for delivery of prime editing enzymes. Using mRNA encoded Prime editor we accomplished highly efficient gene editing (3%) and restoration of mitomycin C resistance. We next established a protocol to expand HSCs (up to 5 x10^5/mouse) from either FancL^{TATdel} adult bone marrow or fetal liver cells in serum-free media. In these *in vitro* expanded HSCs we demonstrated similar editing efficiency (3%) with higher resistance to mitomycin *in vitro* following selection (enriched to 44% of the whole population). We also achieved high levels of *in vitro* editing in other cell types, such as primary and immortalized fibroblasts. In all cases, restoration of just a single allele can restore normal FA/BRCA1 pathway function.Overall, our findings illustrate that a causative FA mutation associated with loss of the FA/BRCA pathway does not prohibit prime editing-mediated gene correction. Corrected cells behave as wild-type in all *in vitro* assays tested, and *in vivo* transplantation experiments are ongoing. The FancL^{TATdel} mouse model is the first model of FA with an human patient mutation, and will be a useful pre-clinical model to further explore the potential of gene editing in the treatment of bone marrow failure syndromes.

510 Circular Single-Stranded DNA is a Superior Homology-Directed Repair Donor Template for Efficient Genome Engineering

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The toolbox for genome editing in basic research and therapeutic applications is rapidly expanding. While efficient targeted gene ablation using nuclease editors has been demonstrated from bench to bedside, precise transgene integration remains a technical challenge. AAV6 has been a prevalent donor carrier for homology-directed repair (HDR) mediated genome engineering but has reported safety issues, manufacturing constraints, and restricted applications due to its 4.5 Kb packaging limit. Non-viral targeted genetic knock-ins rely primarily on double-stranded DNA (dsDNA) and linear single-stranded DNA (lssDNA) donors. Both dsDNA and lssDNA have been previously demonstrated to have low efficiency and cytotoxicity. Here, we developed a non-viral genome writing catalyst (GATALYST^{TM}) system which allows production of ultrapure, minicircle single-stranded DNAs (cssDNAs) up to ~20 Kb as donor templates for highly efficient precision transgene integration. cssDNA donors enable knock-in efficiency of up to 70% in induced pluripotent stem cells (iPSCs), superior efficiency in multiple clinically relevant primary cell types, and at multiple genomic loci implicated for clinical applications with various nuclease editor systems. When applied to immune cell engineering, cssDNA engineered CAR-T cells exhibit more potent and durable anti-tumor efficacy than those engineered from AAV6 viral vectors. The exceptional precision and efficiency, improved safety, payload flexibility, and scalable manufacturability of cssDNA unlocks the full potential of genome engineering with broad applications in therapeutic development, disease modeling and other research areas.

511 lgRNA-Guided Precise CRISPR Gene Editing for the Cure of Chronic HBV Infection

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Prior studies reported in the literature support the possibility of eliminating hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) by the clustered regularly interspaced short palindromic repeats (CRISPR) gene editing for the cure for chronic hepatitis B. However, the achievement of this therapeutic goal requires further improvement of the CRISPR editors and hepatocyte-targeted delivery technologies. Specifically, the cure of chronic HBV infection requires high in vivo editing efficiency to eliminate or inactivate the vast majority of cccDNA to allow the ultimate clearance or complete control of residual viral infection by human immune responses. The off-target editing, as well as on-targeting cleavage of integrated HBV DNA in cellular chromosomes, introduce unintended genetic changes in patients. In addition, due to the relatively low editing efficiency, multiple doses of current CRISPR/Cas therapeutics are most likely required for significant reduction of the cccDNA pool in the liver, which may be limited by the immunogenicity of the CRISPR

ribonucleoprotein (RNP) complexes and of their delivery vehicles such as adeno-associated viruses (AAV). Improvement of guide RNAs is of crucial importance for advancing CRISPR gene editing for the treatment of diseases, including chronic HBV infection. Chemically ligated guide RNA (lgRNA) is a next-generation single-molecule guide RNA (sgRNA) for precise gene editing by CRISPR-associated proteins (Cas). LgRNAs are produced by robust, convergent, and scalable chemical synthesis via efficient chemical ligations of short RNA segments readily accessible by solid phase synthesis. By this method, 196-mer lgRNAs were synthesized. This new technology not only makes manufacturing long RNAs cost-effective but also gives access to high-quality validated full-length products with much fewer synthetic errors at the critical spacer segment than classic sgRNA. Obviously, it enables cost-effective global chemical modifications for better efficacy, selectivity, and stability, as well as targeted delivery by molecular tagging and other formulation technologies. Thus far, we have synthesized and evaluated more than 50 ultra-pure chemically modified lgRNAs. CRISPR-Cas9 guided by these lgRNAs efficiently cleaved target DNAs in vitro and edited HBV cccDNA as well as integrated HBV DNA in human hepatoma cells supporting HBV replication and gene expression. Particularly, our cell-based studies showed that a single transfection of one single lgRNA edited more than 80% of cccDNA and integrated HBV DNA and reduced the levels of HBV surface antigen (HBsAg) and e antigen (HBeAg) by approximately 90%, targeting at various regions of HBV genome respectively. One highly potent lgRNA was identified (99% reduction in both HBsAg and HBeAg levels). Progress in the development of an lgRNA-guided STAR (Search-Tag-Amend-Release) editing platform to overcome the existing barriers to HBV DNA editing therapeutics will also be presented.

512 Environment Social Governance Solution for Fluorescence-Labeled ssDNA Fragment Analysis Using Denature Microfluidics Capillary Electrophoresis, an Application for CRISPR Enzyme Study

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For research use only. Not for use in diagnostic procedures. **Introduction:** Capillary electrophoresis (CE) and microfluidic CE (MCE) have been widely used in nucleic acid fragment analysis. In studies of nucleic acid modification enzymes such as CRISPR enzymes, PCR amplification analysis, where one strand of nucleic acid substrate needs to be analyzed, denaturing CE has been the common method. We have demonstrated the formamide denaturing MCE to characterize CRISPR-Cas9 genome-editing efficiency using fluorescence-labeled fragments as the substrate. Although formamide is a standard nucleic acid denaturing chemical, it is a reproductive toxin and is regulated as "danger" in Safety Data Sheet (SDS), with stringent exposure limits by EU regulatory guidelines. Besides, artifact peaks due to incomplete denaturation often occur in denaturing CE, which interfere with the accuracy of fluorescence-labeled ssDNA analysis. This study was designed to evaluate an environment green denaturing chemical and a new method for reducing artifact peaks in MCE. **Method and Result:** PerkinElmers platforms, LabChip® GXII Touch™ (Part# CLS138160), FFA 120 reagent (Part#: CLS158081), and DNA 5K/RNA/CZE HT LabChip (Part#:760435) were used in the study. The artifact peaks were observed in MCE with DMSO as the denaturing reagent (Figure 1). Mainly two peaks are observed for the 3 tested PCR products (120bp, 200bp, 500bp) prepared with one primer labeled by Cy5 at 5' end (Figure 1A). The slower-migrating peak is the ssDNA product, and the faster-migrated peak is the dsDNA product. After the Lambda exonuclease treatment of digesting the strand without fluorescence labeling and purification, only the slower-migrating ssDNA peak is detected (Figure 1B). The dsDNA artifact peaks can be reduced by adding chelators e.g. EDTA (Figure 2). The peak percentage of dsDNA artifact is reduced substantially with the increase of EDTA. When the EDTA amount is equal to or higher than the magnesium concentration in the sample (4mM), there is no obvious difference (over 5%) of heat treatment on further reducing artifacts. **Conclusion:** This study proves that DMSO, which is nontoxic, and recyclable, can be used as a denaturing reagent for MCE system and the dsDNA artifact peaks can be reduced by adding a certain amount of EDTA into the sample without the need for additional heat denature. As an Environment · Social · Governance (ESG) solution, it has direct, positive impact on the environment and reduces hazardous waste from daily research.

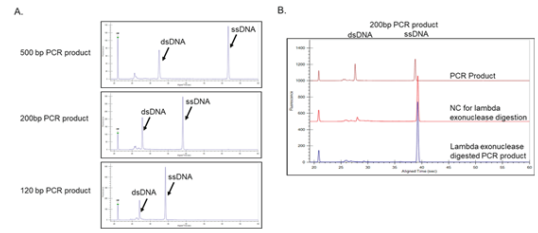

Figure 1: dsDNA artifact peaks in DMSO-mediated denaturing MCE system. A. 3 PCR products; B. 200bp PCR product with different treatments.

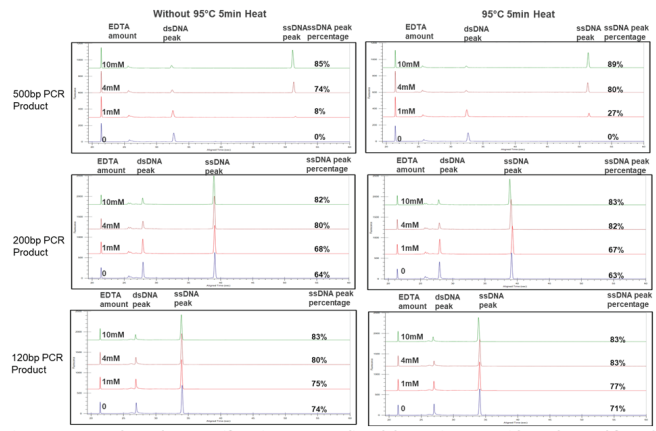

Figure 2. EDTA reduces dsDNA artifacts in DMSO-mediated denaturing MCE without the need for 95°C heat denature.

513 Pin-point™ Base Editing System: A Versatile Editing Platform Driving Cell Therapies

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Gene editing technologies have successfully been used to improve the next generation of cell and gene therapies. However, standard gene editing platforms such as CRISPR-Cas9 trigger concerning cell toxicity and off-target effects due to the formation of DNA double-strand breaks (DSBs) that could hinder wider clinical applications. Base editing offers an ability to correct disease-causing mutations or knock out genes in a multiplexed manner, without introduction of DSBs. The first cohort of base editing therapeutics entered clinical trials only a few years after the development of the technology. Horizon's modular Pin-point base editing system, uses a nickase Cas9 with an aptameric guide RNA to recruit a deaminase to the site of interest, facilitating highly efficient and precise nucleotide conversion. We optimized design and delivery conditions of chemically modified synthetic guide RNAs and enzymatic mRNA to apply multiplex editing with our Pin-point base editing system to the development of engineered CAR-T cells. We target a set of therapeutically relevant loci for the development of allogeneic CAR-T cells achieving high knockout efficiency and editing purity at all sites simultaneously with a safer editing profile and enhanced cell viability compared to traditional nuclease systems. Our technology also enables robust simultaneous targeted knock-in and multiplex knockout without the requirement of additional sequence-targeting components. The ability to perform complex genome editing in multiple cell types (such as T cells, iPSCs, HSCs) safely, efficiently, and precisely opens the door to the application of the Pin-point system in a range of advanced cell therapies.

514 CRISPR/dCas9 Mediated Epigenetic Modifications in CASK-Related Disorders

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CASK-related disorders are caused by mutations in an important regulatory gene in the brain, calcium/calmodulin-dependent serine protein kinase (CASK) and can result in microcephaly with pontine and cerebellar hypoplasia (MICPCH), in addition to intellectual disability, and seizure activity. CASK-related Intellectual Disabilities (CASK-ID) predominantly affect the female population and are classified as X-linked neurodevelopmental disorders. CASK-ID is typically the result of a *de novo* loss of function mutation. As females have two X-chromosomes, disease causing mutations present with a 50/50 expression of mutant and wildtype, due to the mosaicism caused by random X-chromosome inactivation (XCI). This mosaicism presents an interesting therapeutic option of reactivating the intact allele in affected neurons.Previously, the Fink lab has shown the ability to target *CDKL5* in human neuron-like cells and reactivate the XCI-silenced allele using our CRISPR/dCas9 epigenetic approach. Our current goals are to optimize this approach with a smaller split-CRISPR orthologue, sadCas9, to meet the packaging limit of an Adeno-associated Virus (AAV). We also aim to apply this epigenetic rescue technology in disease relevant cell lines and eventually in patient-derived iPSC neurons. We aim to accomplish this through cloning and testing sadCas9 with identified guide RNAs to target the *CASK* promoter. Constructs will be tested for optimal targeting efficacy and assessed through gRNA screens measured via RT-qPCR. Additionally, epigenetic modifications from our approach will be analyzed through bisulfite sequencing. Currently, the lead guide RNAs were evaluated in HEK293Ts and fibroblasts that contain a coding SNP to measure reactivation.Our results show the ability to target *CASK* and assess gene expression changes with sadCas9 and paired epigenetic modifier. This shows the potential for sadCas9 to be utilized for epigenetic editing and the potential for a therapeutically relevant delivery of the CRISPR/dCas9 system. This project will allow for a therapeutic platform that can be more readily packaged in an AAV delivery method and applied to CASK-related disorders.

515 Molecular Dynamics of Genome Editing with CRISPR/Cas9 and rAAV6 Virus in Human HSPCs to Treat Sickle Cell Disease

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Since the discovery of CRISPR/Cas9 system in 2012 as a tool to make DNA double-strand breaks, genome editing has exploded as a research tool. Moreover, gene therapy with CRISPR/Cas9 has risen as a promising and powerful therapeutic approach for many diseases which previously were untreatable or for which suboptimal treatments were available, particularly in genetic disorders. Currently, an increasing body of clinical trials have been opened worldwide with CRISPR/Cas9 system operating a variety of genomic modifications, and promising results of safety and efficacy have been achieved in some of these clinical trials. A better understanding of molecular changes and cellular responses during and after CRISPR/Cas9 genome editing *ex vivo* in clinically relevant cells is vital to ensure the progress and success of this technology. Sickle cell disease (SCD) is the most common serious monogenic disease with 300,000 births annually worldwide. It is an autosomal recessive disease resulting from a single nucleotide variant in codon six of the β-globin (HBB) gene. Currently, the only curative treatment of SCD is allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, HLA-matched donors are limited, and graft-vs-host disease (GVHD) may occur after allo-HSCT. *Ex vivo* gene correction in autologous hematopoietic stem/progenitor cells (HSPCs) with CRISPR/Cas9 and rAAV6 (a recombinant adeno-associated virus serotype 6) virus as DNA donor template to treat SCD has now entered early-phase clinical investigation with the aim of proving feasibility, safety and efficacy of editing the disease-causing mutation in HBB gene with CRISPR/Cas9 and rAAV6 (NCT04819841). To better understand the molecular mechanism of genome editing with CRISPR/Cas9 and

rAAV6, and to ensure the safety of genome edited drug products, we developed sensitive assays for detecting changes of manufacturing reagents and for cellular responses during and post genome editing. In this study we demonstrated 1) The remarkable stability and activity preservation of the rAAV6 donor template virus after multiple freeze/thaw cycles or storage at 4°C or storage at room temperature. 2) Better genome editing outcomes when frozen mobilized peripheral blood HSPCs were pre-stimulated for 72 hours compared to 48 hours with better cell survival and higher number of gene corrected cells without compromising stem cell properties and engraftment efficiency. 3) The transient activation of p53 pathway in human HSPCs invoked by all genome editing machinery components peaking at 6 hours post genome editing treatment and remaining partially activated at 48 hours post genome editing. 4) Dynamic changes of rAAV6 capsid and Cas9 proteins during and after HBB gene editing with no detectable residual rAAV6 capsid proteins and a trace amount of residual Cas9 protein in the cells at the completion of manufacturing. These findings will be beneficial to better understanding the molecular mechanism of *ex vivo* genome editing in HSPCs, and they will also provide important insights into the development and application of CRISPR/Cas9/rAAV6 genome editing and highlight some of the current safety and efficacy properties of the process.

516 Newly Discovered and Engineered CRISPR-Associated Nucleases as a Robust, Efficient, Allele- and Target-Specific Editing Tool

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CRISPR-based gene editing holds great promise for curing various medical conditions. However, several limitations in current CRISPR-Cas systems restrict harnessing the full potential of genome-wide editing. First, commonly used nucleases share a narrow repertoire of PAM recognition motifs, limiting the range of genomic targets. Second, editing efficiency is occasionally insufficient to achieve the desired goal. Third, off-target effects remain a major concern in most CRISPR-Cas systems. In particular, achieving allele-specific editing, required for treating diseases caused by dominant negative mutations, is a highly challenging goal. To address these challenges, we first used *in silico* analysis of metagenomic databases, and discovered a large number of novel nucleases that are active in mammalian cells. Importantly, these nucleases have diverse PAM recognition domains, thereby enabling gene-editing of genomic sequences that are inaccessible by commonly used nucleases. Our unique engineering platform enabled us to further optimize one of our leading nucleases, which already showed intrinsic high fidelity and activity when tested in mammalian cells. This resulted in improved variants that perform highly efficient, allele-specific editing - up to a single nucleotide difference - with no detectable off-targets. These proprietary engineered variants are currently under clinical development for an *ex vivo* gene editing strategy for severe congenital neutropenia (SCN), which involves specific excision of the disease-causing mutant allele of the *ELANE* gene. Our results demonstrated that monoallelic *ELANE* knockout, using our engineered nuclease, alleviated the major SCN related abnormality in patient-derived hematopoietic stem cells, and resulted in maturation of the edited cells into active neutrophils. Thus, our nuclease discovery and engineering platforms establish a comprehensive approach that offers efficient, high fidelity gene editing at unique targets. This strategy could potentially be available to broad patient populations suffering from a range of genetic disorders.

517 Generative Machine Learning Enables De Novo Guide RNA Design for Precise RNA Editing

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RNA editing mediated by adenosine deaminase acting on RNA (ADAR) unlocks a diverse set of molecular and therapeutic modalities, including the correction of pathogenic G-to-A mutations that underlie many monogenic diseases. The recruitment of endogenous ADAR enzymes by guide RNAs (gRNAs) antisense to a target transcript can allow for precise adenosine-to-inosine (A-to-I, effectively A-to-G) editing at the RNA level. However, the therapeutic potential of this process has been limited due to two factors: ADAR's natural preference to edit adenosines within certain sequence contexts, and its proclivity to edit multiple adenosines in a given dsRNA substrate. Here, we demonstrate the power of combining high-throughput screening (HTS) and generative machine learning (ML) to rapidly engineer novel gRNAs *de novo* to any target of interest. To expand the potential of RNA editing, we sought to design a gRNA capable of recruiting ADAR to selectively edit multiple adenosines. The introduction of three separate A-to-I edits spanning four nucleotides within the coding sequence of APP leads to amino acid substitutions K670G and M671V, which are predicted to decrease BACE1 cleavage and decrease accumulation of amyloid plaques, accumulation of which is a hallmark of Alzheimer's disease. Two of the target adenosines have a 5' guanosine neighbor, a strong editing dispreference of ADAR, adding to the design challenge. Despite an initial screening of nearly 58,000 gRNAs, none were able to edit all three sites simultaneously. However, input optimization on a convolutional neural network (CNN) generated novel gRNAs predicted to have >30% editing across all three sites in tandem, which were then experimentally validated (Figure 1). This generative approach yielded gRNAs with improved tandem editing compared with more traditional sample-and-score "exhaustive" ML approaches. Next we extended generative ML to gRNAs targeting any novel adenosine of interest. We constructed a library of >50,000 gRNAs targeting >5,600 clinically relevant adenosines, with a median of nine gRNAs per target. A target-stratified CNN achieved strong predictive power for editing in genes withheld from the validation set (Figure 2). The model further validated on a separate, single target empirical data set of >20K gRNAs (rho = 0.5-0.7) and allowed us to establish a "target editability" score to flag hard-to-edit adenosines for generative gRNA design prioritization. Overall, our results demonstrate the effectiveness of HTS coupled with generative ML for designing gRNAs for ADAR editing of novel targets.

Ongoing efforts include incorporating transformer architectures of DNA and RNA structure. The ability to rapidly program precise and safe ADAR gRNAs has the potential to significantly advance the development of therapies for monogenic diseases.

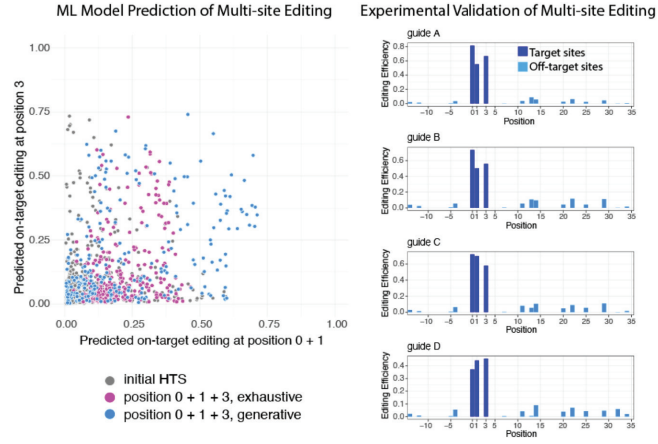

Figure 1 Generative designs identified gRNAs that were predicted and validated to achieve position 0, +1, and +3 co-editing of APP.

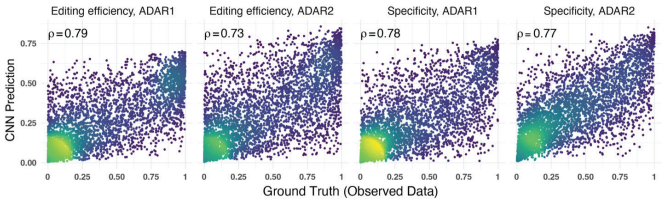

Figure 2 Target-stratified CNNs predict ADAR editing across diverse, withheld targets.

518 Development of Minicircle-Based Non-Viral Delivery of Cytosine Base Editors

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Introduction: CRISPR/Cas has revolutionized genome editing over the past decade. Cytosine base editors (CBEs) can edit DNA without double strand breaks by converting a cytosine (C) base to a thymidine (T) using a cytosine deaminase enzyme linked to Cas9 nickase[1]. A major limitation of all CRISPR technologies is how to safely and effectively deliver them to tissues of interest. The most commonly used delivery tool for BEs are adeno associated viruses (AAVs)[2]. With limited capacity and immunogenicity concerns at high doses, alternative methods for CBE delivery should be explored[2]. Non-viral minicircles (MCs) are derivatives of plasmids that lack a prokaryotic backbone, making them smaller with improved delivery and safety profiles[3]. In this study, we explored the use of MCs for CBE delivery[3]. **Methods:** We engineered parental plasmids (PPs) and MCs containing a third generation CBE or a Cas9 (control) plus appropriate guideRNA (PP-CBE, MC-CBE, PP-Cas9, MC-Cas9). To evaluate BE activity, we employed a previously described activatable reporter gene tool, the Gene On (GO) system[4]. In this, green fluorescent protein (GFP^{GO}) or firefly luciferase ($LUC2^{GO}$) are encoded with a mutated start codon to prevent translation. When the mutation is corrected by a CBE, it allows for direct visualization of BE activity. HeLa cells were transduced with GFP^{GO} or $LUC2^{GO}$ lentiviral vectors to produce stable cells expressing the mutated reporter genes (a constitutive red fluorescent protein was used for FACS). Hela cells were transfected with either PP-CBE, MC-CBE, PP-Cas9, or MC-Cas9. For GFP^{GO} cells, the fluorescent expression of GFP was measured continually for 72 hours using the Incucyte® Live-Cell Analysis Systems. For $LUC2^{GO}$ cells, activity was assessed 48 hours post transfection by bioluminescence imaging (BLI) using a Newton imaging system. **Results:** Substantially more GFP^{GO} cells expressed GFP after transfection with MC-CBE (10.5%) compared to PP-CBE (3%) after 72 hours, and the kinetics of the reaction shows MC-CBE activation occurs more rapidly (Fig 1B). Additionally, transfection of cells with PP-Cas9 and MC-Cas9 showed minimal GFP expression. $LUC2^{GO}$ cells transfected with MC-CBE had significantly higher BLI signal than cells transfected with PP-CBE, PP-Cas9, and MC-Cas9 ($p<0.01$) (Fig 1D). **Conclusions:** Our data supports improved base editing efficiency in cancer cells using MCs encoding both a CBE and gRNA over traditional plasmids. MCs provide an alternative to viral delivery systems and have been associated with excellent safety profiles that might expedite clinical translation. We are currently evaluating the use of MCs to deliver CBEs targeted to genes of interest across a range of cancer cell types both in vitro and in vivo, and for targeted CBE delivery to tissues of interest such as the lungs and liver.

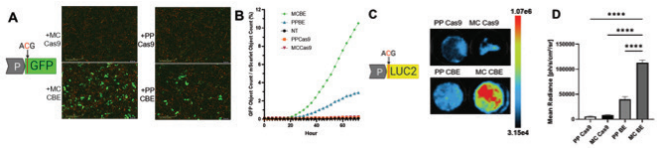

Figure 1: (A) Fluorescence imaging of GFPGO reporter activation (B) GFP object count over 72 hours. (C-D) BLI of transfected LUC2GO cells.

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519 Allele-Specific CRISPR Gene Therapy for Epidermolysis Bullosa Simplex Dowling-Meara with Single-Base Precision

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Epidermolysis bullosa (EB) is a group of rare skin diseases typified by blisters that primarily affect the skin after mechanical trauma. The Dowling-Meara variant of epidermolysis bullosa simplex (EBS-DM) is one of the most severe types of EBS. The major genetic causes of EBS-DM include dominant-negative mutations within either the

KRT5 (*12q13.13*) or *KRT14* (*17q12.q21*) genes. The keratin proteins are highly abundant in the cytoplasm of basal keratinocytes in the epidermis, providing cytoskeletal scaffold within the epithelial cells for protection against mechanical and non-mechanical stresses. These mutations cause keratin 5 and keratin 14 filament network structures to become fragile, which leads to epidermal cytolysis and blistering which are symptoms of EBS-DM. Currently, there are no approved therapy that can cure this disease. Only symptomatic care is available. In this regard, gene therapy might be a potential option for corrective therapy for these patients. To this end, we developed a CRISPR/Cas9 based near-cognate sgRNA method to specifically target the *KRT5* and *KRT14* point mutations to disrupt the dominant-negative mutant form of keratin. We found that this near-cognate sgRNA method efficiently induced genome editing of the mutant alleles without significant effect on the normal keratin alleles. In conclusion, we developed allele-specific CRISPR/Cas9 methods to target *KRT5* and *KRT14* specific mutations associated with EBS-DM.

520 Enhancing the Precision of Designer Site-Specific Recombinases: Methods for Surveying Sequence Specificity and Mitigating Potential Off-Target Effects

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The specific targeting and editing of site-specific recombinases (SSRs) provide an appealing option for gene editing therapies where precision is crucial. Recent developments to engineer and evolve Cre/loxP-type SSRs, have made it possible to shift the specificity of Cre from its native target preference, the palindromic loxP site, to catalyze recombination of a range of DNA substrates, including non-palindromic sites. By taking advantage of the modular nature of the complex, each molecule can be designed to have its own unique specificity, and when expressed together, can form a functional heterotetramer capable of performing DNA-editing reactions on non-palindromic sites from the human genome. Although the ability to combine these evolved molecules into functional complexes drastically broadens the targeting possibilities of SSRs as heterocomplexes, it also carries a risk of unspecific reactions performed by homotetramers formed by individual subunits. To investigate the therapeutic potential of these novel recombinases, it is necessary to profile their general specificity to detect potential off-targets and then apply methods to mitigate any non-specific activity. We developed a high-throughput assay to measure the activity of designer-SSRs on a large set of over 5,000 rationally designed, non-randomized DNA target sequences, which allows building a machine learning model for accurately predicting off-target activity on all possible DNA sequences. The assay is carried out in *E. coli* to minimize experimentation and eliminate artifacts, and employs widely accessible Illumina sequencing as the final readout. We demonstrated the capabilities of this assay by profiling a designer Cre-like complex consisting of two subunits with unique specificities. Although the evolved complex showed high specificity to the on-target sequence, one of the subunits had measurable non-specific behavior when expressed alone. To reduce this effect, we transformed the complex into a functionally obligate system by mutating a single residue in each subunit. The mutated subunits became inactive when expressed alone, and a functional heterotetrametric complex was formed only when the subunits were expressed together. By screening the mutated subunit on a library composed of its potential off-targets, we confirmed its improved specificity. By constructing a DNA specificity model of a SSR, we could determine the best method to mitigate off-targets and improve its specificity, to ultimately use the designer-recombinase complex on a therapeutic target in human cells. Not only do these results facilitate the development of safe and effective therapeutic designer-recombinases but the profiles can also be used to advance our understanding of SSR catalysis.

521 CRISPR-Powered Graphene Electronics-A New Generation of Bioelectronics for CRISPR Quality Control

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Keck Graduate Institute, Cardeabio, CRISPR QC, Claremont, CA

The discovery of CRISPR technology has revolutionized the fields of transcriptional activation and repression, genome editing, gene-based therapeutics, and diagnostics. The applications of this technology have been rapidly expanding as researchers continue to discover new Cas enzymes, engineer high fidelity Cas orthologs, and modify and synthesize guide RNAs to efficiently direct these Cas enzymes to their targets. In this talk, we will introduce the first-generation DNA biosensors that combine CRISPR technology with the ultra-sensitivity of graphene-based field effect transistors (gFETs) to detect target DNA sequences within the whole genome without the need for DNA amplification. This technology, termed CRISPR-Chip™, utilizes the genome searching capability of Cas and reprogrammable RNA molecule to unzip the double-stranded DNA and bind to its target. This binding event causes a change in graphene conductivity which can be detected in real-time within the gFET construct. CRISPR-Chip was first utilized to detect target genes within clinical samples obtained from patients with Duchenne Muscular Dystrophy (Cover of Nature BME-2019), and single cell point mutations in Sickle cell disease and ALS without the need for amplification (Nature BME 2021), within less than 30 minutes. The applications of this technology platform go beyond diagnostics. CRISPR-Chip can provide insight into the factors that impact editing outcomes and greater insights on the mechanism of CRISPR and can lead to safe and more effective utilization of this gene editing technology for therapeutic applications.

522 Large Knock-In in Primary T Cells with Optimized Cas9 HDR Methods and Design

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Integrated DNA Technologies, Coralville, IA

Knock-in of large transgenes in primary T lymphocytes has previously relied on methods that insert via random integration, not allowing for precise editing and making the genome susceptible to insertional mutagenesis (off-target effects, chromosomal rearrangements, translocations, etc.). As such, there is a need for precision gene editing for introduction of stably expressed transgenes such as chimeric antigen receptor (CAR) variants. CRISPR-Cas9 is an expedient tool for

precision editing and can mediate targeted HDR-based insertion. Here we present optimized reagents, donor design, and delivery methods for CRISPR HDR in primary T cells with chemically synthesized short HDR donors for small inserts and PCR-based long HDR donors for knock-in of up to 2kb. These methods and reagents together in primary human T cells achieve high-efficiency precise genome editing with low off-target integration.

523 Reducing the Inherent Auto-Inhibitory Interaction within the pegRNA Enhances Prime Editing Efficiency

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UMass Chan Medical School, Worcester, MA

Prime editing systems have enabled the incorporation of precise edits within a genome without introducing double strand breaks. Previous studies defined an optimal primer binding site (PBS) length for the pegRNA of ~13 nucleotides depending on the local sequence composition. However, characterization of optimal PBS length has been based on prime editing outcomes using plasmid or lentiviral expression systems. Here we report that the inherent complementarity between the PBS and spacer sequence within the pegRNA affects pegRNA binding efficiency and target recognition. Because RNA-RNA duplexes are typically more stable than RNA-DNA duplexes and due to the intramolecular nature of the association between the spacer and PBS regions of the pegRNA, the formation of a PBS-spacer RNA duplex can preclude the formation of an R-loop by the prime editor at its target site. Finding the correct balance between PBS length and pegRNA sequence composition is critical to maximize prime editing activity by reducing the inherent "auto-inhibition" within the pegRNA sequence composition. We demonstrate that reducing the complementarity between the PBS and spacer sequences by reducing PBS length can broadly improve prime editing activity in the context of prime editor protein complexed with synthetic, end-protected pegRNAs across multiple target sites and multiple cell types. The difference in PBS length requirement between plasmid expression systems and PE RNPs loaded with synthetic, end-protected pegRNAs appears to originate from the susceptibility of transcriptionally produced pegRNAs to 3' end degradation. By small RNA sequencing, we observe that while the majority of the transcriptionally produced pegRNAs are full length, the pegRNA species that are bound by the prime editor protein are enriched for 3' truncated species. In the case of end-protected pegRNAs, we demonstrate that shorter PBS lengths with a PBS-target strand melting temperature near 37°C are optimal. Additionally, a transient cold shock treatment of the cells post PE-pegRNA delivery further increases prime editing outcomes. Finally, we show that prime editor ribonucleoprotein complexes programmed with pegRNAs designed using these refined parameters efficiently correct disease-related genetic mutations in patient-derived fibroblasts and install precise edits in primary human T cells and zebrafish.

524 Cytosine Base Editing of Surfactant Protein C Mutation Restores Genotype and Phenotype in Human and Murine Models

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RATIONALE Mutations in the surfactant protein C (*SPC*) gene can lead to lethal neonatal respiratory distress syndrome or pediatric interstitial lung disease. The most common *SPC* mutation is a thymine-to-cytosine base change that leads to a substitution of threonine to isoleucine at codon 73 (I73T), resulting in mistrafficking of proSPC protein and blockage of cellular macroautophagy. Cytosine base editing allows for safe, site-specific conversion of cytosine to thymine without double stranded breaks. Here, we demonstrate *in vitro* genotypic and phenotypic correction of the SPC I73T mutation by cytosine base editing in both human and mouse *SPC*. METHODS Human embryonic kidney cells (HEK293) constitutively expressing either mutant (huSPC-I73T) or wildtype (huSPC-WT) human SPC cDNA were treated via nucleofection with a combination of cytosine base editor (CBE) mRNA and custom guide RNAs (sgRNA) specific to the I73T mutation site. Gene editing efficiency was determined by Sanger sequencing, and the top performing sgRNA/CBE were used to assess phenotype rescue. Differences in proSPC trafficking before and after correction were analyzed by Western blot of HEK293 cell lysate and immunofluorescent microscopy of fixed cells. Correction of macroautophagy blockage was assessed by Western blotting for LC3B, a marker of autophagosome turnover. A similar guide screen was conducted in HEK293 cells expressing the murine SPC mutation (muSPC-I73T) prior to encapsulation of the top performing murine sgRNA and CBE mRNA into lipid nanoparticles (LNPs) for non-viral delivery to cells. RESULTS Nucleofection of hSPC-I73T cells achieved up to 95% correction of the I73T mutation. Western blot confirmed mistrafficking of SPC protein in unedited huSPC-I73T cells as demonstrated by an inappropriate accumulation of intermediate isoforms of proSPC. After cytosine base editing, aberrant proSPC banding proSPC was resolved. Immunofluorescent microscopy of fixed, unedited huSPC-I73T cells demonstrated abnormal accumulation of immature SPC protein in the plasma membrane and throughout the cytoplasm. After cytosine base editing of huSPC-I73T cells, immunofluorescent microscopy showed punctate patterning of proSPC without plasma membrane accumulation, consistent with the appearance of huSPC-WT cells and correction of the trafficking defect. In addition, cytosine base editing of huSPC-I73T cells resulted in reduction of intracellular LC3B accumulation, suggesting resolution of the macroautophagy defect present in unedited cells. In muSPC-I73T cells, nucleofection with the top sgRNA/CBE mRNA achieved 60% correction of the I73T mutation. Although further characterization of base edited muSPC-I73T was not pursued since these cells lack a disease phenotype, a translatable LNP gene delivery platform encapsulating the sgRNA and CBE mRNA

was engineered and applied to achieve 30% correction of the murine I73T mutation. CONCLUSION Cytosine base editing is a promising therapeutic strategy for genetic diseases caused by point mutations, including the SPC family of congenital diseases. In this study, we demonstrate correction of the SPC I73T mutation with subsequent return to physiologic protein trafficking and macroautophagy in human cells. This discovery provides the framework for future in vivo base editing studies with LNPs to ultimately develop a novel therapy for pediatric patients with SPC mutation disease.

525 Engineering a Potential Curative Treatment for Hemophilia A Using an AAV Dual Targeting Strategy

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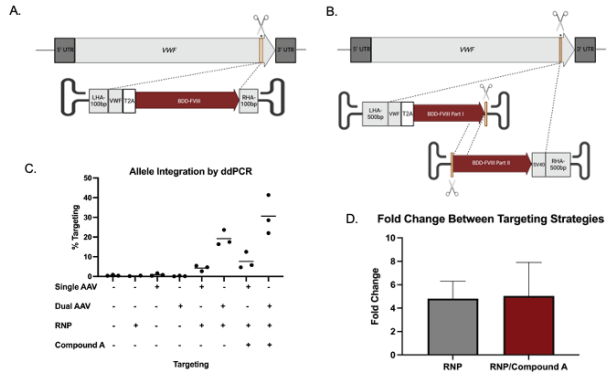
Hemophilia A is an X-linked bleeding disorder that effects 12 in every 100000 assigned male at birth within the United States. Within this subpopulation almost half have the severe phenotype, with less than 1% Factor VIII activity, correlating to spontaneous bleeding among other symptoms. Current treatments can be costly and needed across the lifetime of the patient with no FDA approved cure available. Here we report an efficient CRISPR/Cas9 editing strategy using an AAV6 donor template which expresses a B domain deleted Factor VIII (BDD-FVIII) gene cassette to provide a potential curative treatment for Hemophilia A. In our approach we selected the von Willebrand Factor gene locus, an endogenous platelet locus within the hematopoietic lineage, for protein expression limited to the megakaryocyte/platelet cell type. Our novel strategy uses a dual AAV cassette targeting approach. This strategy follows the idea that a dual targeted construct integrates simultaneously for an increase in the targeting efficiency at the locus. Our dual vector system has integration at a frequency of 20-30% compared to the single AAV vector with integration of 5-10%. The targeted cells show Factor VIII expression in CD41+/CD42a+ in vitro differentiated megakaryocytes and maintain multilineage differentiation potential in-vitro.



526 SPOT-ON is a Cas9 Nuclease with No Detectable Off-Targets and Reduced Chromosomal Translocations *In Vivo*

Sandra Wimberger, Dmitrii Degtev, Burcu Bestas, Alexandra Madsen, Aleksandar Zdravkovic, Michelle Porritt, Pinar Akcakaya, Marcello Maresca, Grzegorz Sienski

AstraZeneca, Mölndal, Sweden

The CRISPR-Cas9 systems have revolutionized the field of genome medicines. However, only a few Cas9 enzymes work efficiently in the in vivo setting and even fewer made it to clinical trials. While the first enzyme, SpCas9 has been successful in early trials, its aberrant activity raises safety concerns due to off-target editing. Here, we describe the discovery of a new Cas9 member of the Type II-B family from the human gut microbiome metagenomic database, which we named SpOT-ON. We characterized SpOT-ON biochemically and showed that its gene editing activity in vitro and in vivo is comparable to SpCas9. Remarkably, SpOT-ON displayed intrinsic high-fidelity and undetectable levels of off-target editing evaluated by conventional methods and high-sensitive DUPLEX-sequencing. Genome editing with SpOT-ON results in reduced levels of genomic translocation compared to SpCas9. Overall, SpOT-ON could serve as an alternative to SpCas9 for clinical use with a significantly improved safety profile. At the ASGCT Annual Meeting, we will present the discovery and engineering of SpOT-ON and our latest efforts to further improve its editing properties. Furthermore, we will share the results of in vivo studies using SpOT-ON at AstraZeneca.

527 Characterization and Quantification of CRISPR/Cas9-Induced Genomic Alterations and AAV Targeted Integration in Human HSPC

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CRISPR/Cas9 technology has revolutionized our ability of genome editing and is a very promising tool for gene therapy. However, recent studies show that it can result into extensive on-target damage (extended deletions, chromosomal truncations, chromothripsis) in edited human cell lines, primary cells or human embryos. In addition, little is yet known about the CRISPR mediated targeted integration outcomes in primary cells. Here, we carry out a detailed analysis of the genomic events occurring upon CRISPR/Cas9-based gene editing, alone or in combination with the targeted integration of a therapeutic viral vector in HUDEP-2 cell line and clinically relevant Hematopoietic Stem/Progenitor Cells (HSPCs). To that end, we perform long read sequencing of a targeted genomic region of interest (ROI), using the Oxford Nanopore Technology (ONT) and the recently developed Cas9-based enrichment procedure. This approach allows us to enrich for our ROI avoiding PCR amplification biases. We choose to target α-globin locus, as we have previously shown it is a genomic safe harbour site for editing and targeted integration in human HSPCs, with Cas9 (nuclease and nickase)-gRNA ribonucleoprotein complexes alone or in addition with an adeno-associated virus (AAV) encoding for β-globin

gene, flanked by homology arms for homologous recombination. Noteworthy, our single gRNA targets both HBA1 and HBA2 genes and can result in HBA2 deletion which allows us to simultaneously assess the editing occurring after gRNA single cut as well as the genomic deletion. We perform Cas9-enrichment of a 14kb ROI to be able to recover most possible editing events with statistical significance (~100X coverage) and we establish a bioinformatical pipeline to process the generated data and explore the structural variations. We first focus on the effect of gRNA cut. We show that large unintended deletions (> 500bp) at the gRNA site as well as the deletion of the HBA2 locus occur at higher frequencies in HSPC (40%) than in HUDEP-2 cells (25%). These events are decreased with the use of Cas9 nickase. Then, we analyse cells with targeted DNA integration. We observe that AAV integration efficiency is also cell type dependent, with higher efficiency obtained in HSPCs (70%) than HUDEP-2 (20%), suggesting that these widely used cell lines are not ideal as model for genome editing. Interestingly, most of the integrations are HDR mediated but cases of AAV ITRs on-target-trapping are detected probably due to non-homologous end joining. Unexpectedly, we reveal the existence of complex rearrangements of integrated AAV genomes, concretely concatemers accounting for 20% of the AAV integrations. ITRs are also detected in AAV concatemers integration (on the junctions of the copies). We confirm our results by independent approaches as ddPCR, for the quantification of HBA2 deletion and of HDR mediated AAV on-target integration frequency, and FACS analysis for the quantification of on and off-target integration of the GFP-cassette. We are currently studying the effect of commonly used drugs affecting DNA repair on the editing and integration outcomes. In addition, we are performing ONT sequencing on edited single cell clones to further confirm our observations in bulk populations. Similar analyses are on-going for additional genomic targets and vectors. Our findings provide a clear understanding of the on-target genomic alterations occurring upon CRISPR editing and targeted integration of AAV vectors in the HSPCs, using Cas9 nuclease, nickase and drugs acting on DNA repair. This knowledge is important for future clinical applications of the CRISPR gene editing tool.

528 LNPs for Editing NASH Patient Liver Derived Organoids

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Background: Patient derived organoids (PDOs) that partially recapitulate the 3D structure(s) of an organ or tissue are increasingly seen as crucial tools to model diseases and develop human relevant therapeutics because they are direct descendants of a patients adult stem cells located in the diseased organ or tissue. The relative lack of fast and efficient gene editing technologies for PDOs, which usually do not divide after their final differentiation, is still a serious drawback preventing their full implementation. LNPs complexed with Cas9 mRNA/gRNA have recently emerged as powerful gene editing tools for animals and humans but despite their promise have not yet been systematically used for editing PDOs. LNPs are uniquely capable to deliver mRNA of up to 12kB into non-dividing cells and allow unperturbed safe repeat applications in organoid culture as well as the clinic as they induce the immune system to manageable levels. For many types of PDOs it is not yet clear how well LNP mRNA complexes work, in part since it is unknown under what conditions PDOs can take up the LNPs at an efficiency that is similar to the parental tissue. **Rationale:** For proof of principle that LNP mRNA complexes can efficiently target PDOs, we decided to set out with targeting non-alcoholic steatohepatitis (NASH) patient liver derived organoids (NASH-PDOs). We have recently described NASH-PDOs derivation from human NASH explant livers and their surprisingly high utility in modeling human NASH (McCarron et al. Hepatology, 2021). NASH is a common liver disorder, but genetically complex and heavily influenced by non-genetic factors including nutrition, exercise and infection, making it difficult to define therapeutic targets. Thus, establishing LNP mRNA complex - mediated gene editing in NASH-PDOs would dramatically improve their ability to functionally probe candidate NASH genes and pathways. **Results:** Using GFP mRNA LNPs in a 96 well 3D culture format with IF microscopy as output we have developed a method resulting in > 90% efficient targeting of total cells in the terminally differentiated non-dividing NASH-PDOs 2-3 days after addition of the LNP GFP mRNA complexes. This is an order of magnitude better than any other known targeting method for most types of PDO. These NASH-PDOs mostly contain hepatocyte- and cholangiocyte - like cells which are both transfected by the GFP mRNA LNPs. **Conclusions:** Our GFP mRNA LNP based results suggest that LNP mediated Cas9 mRNA/gRNA gene editing or mRNA mediated transgene over expression will be highly efficient in non-dividing terminally differentiated NASH-PDOs. We are currently employing Cas9 mRNA based gene editing using this system. We are also working on developing selective LNP targeting strategies for NASH liver derived macrophages, myofibroblasts and endothelial cells, all of which play important roles in NASH pathogenesis. Since these cells are involved in all types of liver diseases the implications for such liver cell type specific LNP-mediated targeting could be far reaching.

529 Impact of Three-Dimensional Spatial Genome Organization on Gene Editing and Its Use in Gene Editing Drug Design for Higher Safety

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CRISPR-Cas technology is promising in providing targeted gene editing and modulation of the gene expression for therapeutic outcomes. However, there are still technical challenges to be addressed to bring it widely into medical applications including potential off-target effects. The off-target effects include unintended genome edits and rearrangements by the repair mechanism, which could lead to serious deleterious outcomes including carcinogenesis. While the application of CRISPR-Cas in clinical settings is rapidly advancing, our understanding of how the editing technology interacts with the genome and its consequences- both short and long term is relatively under explored.

The three-dimensional (3D) spatial organization of chromatin plays a critical role in gene regulation and function. Multiple studies reported inter- and intra-chromosomal physical contacts between loci is required to coordinate regulation of multiple genes within specific transcriptional activities. Furthermore, those spatial organization and regulation of the genes are tissue and cell specific. To date, several approaches have been taken to accurately predict off-target effects mainly based on sequence and/or epigenetics data, however, none of them have examined the 3D spatial organization of chromatin and consequent regulatory effects in the context of CRISPR-Cas gene editing with tissue and cell-specific angle. Here, we evaluated how 3D spatial organization affects CRISPR-Cas off-target editing activity, potential translocations and potential regulatory pathway modifications. We compared contact frequency measured by Hi-C assay of the genomic loci between experimentally validated edits and mismatch based predicted off-targets in HeLa cells. Contact frequency between experimentally validated edits were significantly higher than between experimentally validated and predicted but experimentally not validated edits ($p<0.05$), suggesting that chances of editing at off-target site are increased when the loci are in higher contact frequency with the genomic loci with edits. Chromosomal translocations associated with CRISPR-Cas editing bring critical structural and molecular changes in the cell, however it is experimentally expensive to capture the entire effects. Thus, we calculated gene homology between high-contact loci to evaluate the risk of possible translocations in silico. Contact frequency of the translocation sites were also examined. The presented results underscore the importance of considering 3D spatial genome organization and proximity in a tissue and cell-specific manner to understand and reliably predict off targets, and their critical regulatory functional consequences. The approach is essential in developing precision medicine to deliver safer gene editing therapy.

530 ARRDC1-Mediated Microvesicles (ARMMs) as a Novel Non-Viral Modality for Efficient Functional Delivery of Adenine and Cytidine Base Editor Proteins Complexed with Guide RNA

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Significant advances have been made over the past 10 years in the discovery and development of novel genome editing proteins to precisely and safely modify genetic sequence. However, the application of these technologies and the ability to articulate novel *in vivo* therapeutic strategies has been limited by the absence of appropriate delivery vehicles that permit: 1) efficient encapsulation of editors, particularly larger Cas-derived editors complexed with guide RNA (gRNA); 2) transient functional delivery of the genome editing machinery to target cell types; and 3) the ability to re-dose. Here, we report an approach to engineer a class of human vesicles called ARMMs (ARrdc1-Mediated Microvesicles) to load and deliver adenine and cytosine base editors (ABEs and CBEs, respectively) complexed with targeting gRNAs in cultured mammalian cells and *in vivo*. Cas-derived base editors, typically composed of an engineered Cas protein fused to natural or laboratory-evolved deaminase enzymes, have emerged as powerful tools to specifically edit genetic sequence without introduction of double-stranded breaks. We used a suspension producer cell line to produce and engineer ARMMs intraluminally loaded with ABE or CBE complexed with gRNA. ARMMs purified from producer cell cultures were analyzed for ABE, CBE, and gRNA content. Approximately 10-15 complexes were loaded per ARMM indicating a high level of compacting of complexes within ARMMs. We then treated a range of cell types, including primary and post-mitotic cells, with ARMMs loaded with base editors complexed to gRNA. ARMMs mediated dose-dependent editing of targeted genomic loci, achieving near-complete editing levels at higher multiplicities of ARMMs to cells. *In vivo* evaluation of ARMMs in mice yielded increasing levels of editing with repeat administration, reaching ~ 60% editing in a challenging-to-target cell type after two doses. These results suggest ARMMs as a promising non-viral delivery vehicle for base editors.

531 Precise Temporal Regulation (Titratable and Reversible) of Gene Editing with a Small Cas9 Nuclease by an FDA-Approved Small Molecule Drug, Using cytoDRiVE® Technology

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Gene editing has the potential to revolutionize the treatment of many genetic diseases and several *in vivo* gene editing therapies have recently entered the clinic. To increase their safety and efficacy, precise spatial and temporal on-target editing with minimal off-target editing is highly desirable and will require tight intrinsic or extrinsic control of the gene editor. To this end, we have developed small drug-responsive domains (DRDs) as part of our cytoDRiVE® platform that can be fused to Cas9 nuclease enabling precise control of its expression and activity by exposure to a clinically approved small molecule drug. The small size of these DRDs requires minimal vector space and will allow delivery of DRD-Cas9 and guide RNA in a single AAV vector. We show that the DRD-Cas9 fusions have extremely low editing activity in the absence of drug which increases to near constitutive levels of activity upon treatment with activating drug for a panel of guide RNA targets. In addition, we show that fusion of DRDs to Cas9 does not affect target specificity or repair patterns of the nuclease. This level of control demonstrated *in vitro* by our cytoDRiVE® platform supports potential future clinical utility in cell and gene therapies, where tight regulation of DRD-Cas9 would give the physician control of Cas9 activity in the patient. Pausing or stopping administration of the activating drug once edits have been made could limit off target editing activity and reduce the risk of genotoxicity.

532 Altered Kinetics of Immunoreceptor Internalization Following Activation as a Mechanism for Enhanced Anti-Tumor Function of KIR-Based Chimeric Antigen Receptors

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We previously described a chimeric killer immunoglobulin-like receptor (KIR-CAR) approach to modifying T cell antigenic specificity that mimics the multichain design of natural immunoreceptors. T cells modified to express a mesothelin-specific KIR-CAR with the natural ITAM-containing adaptor, DAP12 demonstrate significantly enhanced anti-tumor activity in a pre-clinical model of mesothelioma when compared with traditional CD3z-based CAR designs such as those used in tisagenlecleucel or axicabtagene ciloleucel. We have extended these observations to show that T cells expressing a KIR-CAR specific for EGFRvIII also have significantly enhanced antitumor activity in two different orthotropic xenograft models of glioblastoma (GBM), U87-vIII and primary GBM D270, when compared with 2nd generation, 4-1BB-costimulated, CD3z-based CAR T cells previously used in a clinical trial at our institution (NCT02209376). Our prior studies demonstrated that a transient loss of CD3z-based CAR expression is associated with CAR T cell hypofunction in vivo in tumor-infiltrating lymphocytes. We hypothesized that the retention of function in KIR-CAR TIL is related to a reduced rate of receptor internalization following receptor activation, compared with standard CD3z-based CAR T. In order to evaluate CAR internalization kinetics, we utilized a surface protein tagging method using the SpyCatcher-SpyTag™ system in which the CAR ectodomain is comprised of a Myc-tagged-SpyCatcher domain and the antigenic specificity of the CAR is conferred by addition of a fluorescently-labeled SpyTag-DARPin fusion protein (SpyDARPin) that specifically binds the SpyCatcher domain covalently via its SpyTag and recognizes the EGFR antigen. A SpyCatcher KIR-CAR (SpyKIR-CAR) or CD3z-CAR with CD28 costimulatory domain (Spy-CAR) were expressed in CD3/28 activated primary human T cells by lentiviral transduction, and their expression confirmed by flow cytometry using an antibody to the myc-tag. Addition of the SpyDARPin exhibited uniform labeling at the plasma membrane as assessed by image cytometry and confocal microscopy. Functional testing demonstrated that the Spy-KIR-CAR and Spy-CARs exhibited comparable cytokine secretion in response to EGFR+ target cells and similar cytotoxicity against EGFR+ U87 glioma target cells in the presence of SpyDARPin. No cytokine secretion or cytotoxicity was observed with SpyCAR and SpyKIR-CAR T cells in the absence of the SpyDARPin. The kinetics of SpyCAR or SpyKIR-CAR fluorescence loss were similar in the absence of EGFR ligand with a half-life of 2 days, consistent with normal membrane protein turnover by pinocytosis. Upon ligand-induced activation, the SpyCAR fluorescence rapidly reduced to background levels of unlabeled SpyCAR cells within 8 hours of activation. In contrast, the mean SpyKIR-CAR fluorescence, while exhibiting a 20% reduction in mean baseline fluorescence at 8 hours, remained increased 4-fold above the background fluorescence. Confocal imaging confirmed the retention of labeled KIR-CAR at the plasma membrane over the time frame in which the labeled SpyCAR at the plasma membrane is lost. We also observed that a substaintial proportion of intracellular CARs appeared to colocalize with lysosomes in the activated SpyCAR T cells, but not SpyKIR CAR T cells. These observed differences in internalization kinetics between the KIR-CAR and CD3z-CAR when coupled with metabolic stress of the tumor microenvironment may explain the ability of KIR-CAR TIL to retain CAR expression and function. Our data also suggests that the retained surface expression of KIR-CAR may be at least partially responsible for the improved antitumor function observed in vivo in solid tumor models.

533 Outcomes from the First NIST Genome Editing Consortium Interlab Study

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Genome editing is being actively pursued globally to transform medicine and bioscience to enable previously impossible advances in cell & gene therapy. Moving the promise of these technologies into production and medical practice requires robust quantitative assays, with associated controls and data tools. **The U.S. National Institute of Standards and Technology (NIST) Genome Editing Consortium (GEC) convenes experts** across academia, industry, and government to collaboratively address precompetitive genome editing measurements and standards needed to increase confidence in evaluating genome editing and utilizing these technologies in research and commercial products. As a result of NIST GEC led efforts there is now a first international standard for genome editing terminology: International Standards Organization- ISO 5058-1:2021 Biotechnology — Genome editing — Part 1: Vocabulary **This presentation will detail the design and results of this first NIST GEC Interlab Study**. The NIST GEC has completed a first of its kind interlab study with organizations in the genome editing field to understand the performance of DNA detection technologies of interest for confirming on- and off-target genome editing. For this interlab study, NIST designed mixture schemes consisting of DNA- or cell-based control samples. These control samples were qualified with ddPCR and NGS to evaluate the accuracy of the study participants' reporting on the size, sequence, and frequency of the DNA variants. DNA variant benchmarks within control samples ranged in size from single nucleotide variants (SNV) to insertions and deletions tens of kilobases long. Variants were present within the interlab study control samples at all of 5 primary frequency bins: 0%, >30%, 5 - 10%, 0.5 -2%, and 0.1 - 0.25%; with two optional samples of ~0.01% and ~0.001%. Participants were provided 5 required samples and a core list of 39 genomic positions of interest to be analyzed by any type of DNA detection process being utilized by the interlab participant. Interlab participants were blinded as to variant sequence and variant frequency within each sample and at each position of interest. **14 Interlab participants returned their assessments** of the samples they were given, including metadata describing the assay and data analysis approach, raw data files, and a list of variants detected- complete with variant positions, sequences, and frequencies. One to two technologies were assessed per participant, with 1-4 replicates per technology.

Technologies assessed include: bulk DNA short read NGS, bulk DNA long read NGS, single cell targeted NGS, genome wide DNA imaging, targeted DNA probe imaging, and capillary electrophoresis fragment analysis. NIST evaluated the results from individual technologies for accuracy of variant detection and frequency prediction. While no one technology had the capability to detect all of the variants at all of the frequencies and sizes, combined results from all of the technologies evaluated, confirmed the ability to measure a subset of variants down to 0.001% and variant sizes from SNV to >100kb. Trends in performance were identified as well as instances where variant signal was present but not reported by the bioinformatics process. **The NIST GEC is working on** a database where all datasets, metadata, and results could be made publicly accessible. Further in-progress NIST GEC work includes development of metadata norms and clonal engineered cell lines to be used as DNA/cell based controls supporting greater assay confidence for the genome editing community and development of genome edited cell based therapies.

534 Discovery and Engineering of a Miniature CRISPR-Cas Type V-L System

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Employing a metagenomic search, we identified a family of miniature CRISPR-Cas type V-L systems capable of RNA-guided dsDNA target cleavage without a tracrRNA. A bacterial depletion screen revealed several active systems which were subsequently shown to have low-level editing in mammalian cells. Among the systems with activity in mammalian cells, we selected a 745aa nuclease effector as a candidate for engineering. An unbiased mutational scanning approach was applied to identify single substitutions that increase indel activity in mammalian cells. Indel-enhancing single substitutions were then screened in combination, with a combinatorially engineered variant demonstrating indel editing activity comparable to SpCas9 in HEK293T cells and SaCas9 in primary cells. Structure determination via cryo-EM revealed the domain organization and nuclease mechanism of the ternary complex. Due to its small size and robust editing levels, this novel miniature CRISPR-Cas type V-L system is an attractive therapeutic candidate for single- or dual-guide excision via all-in-one AAV delivery.

535 Alpha-Synuclein Gene Dosage Human Stem Cell Model is a Powerful Tool to Study Neuronal Differentiation and Cellular Stress in Parkinson's Disease

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Background: The accumulation and aggregation of alpha-synuclein protein (a-syn) is a critical event in Parkinson's disease pathophysiology, impairing neuronal function and contributing to dopaminergic neuronal cell death. Clinical cases with alpha-synuclein (*SNCA*) multiplications or deletions indicate that gene expression levels are essential for neurodegeneration and neurodevelopment (M. Torres *et. al.* 2020). The goal of the study is to combine induced pluripotent stem cell (iPSC) technology with gene editing to establish isogenic cellular tools which express dosage dependent *SNCA* gene copy numbers (Zafar *et. al.* 2022). **Methods:** Here, we developed an isogenic *SNCA* gene dosage model using clustered regularly interspaced short palindromic repeats - CRISPR/Cas9 - gene editing to introduce frameshift mutations into exon 2 of the *SNCA* coding region in iPSCs from a patient with an *SNCA* triplication. We generated and characterized clones with different frameshift mutations relating to 4 knock-out (KO), 3 KO, 2 KO, 1 KO, and 0 KO. The characterization included genotyping, pluripotency test, spontaneous *in vitro* differentiation, karyotyping, and off-target nuclease analysis. Isogenic lines with 0 KO, 2 KO, and 4 KO were differentiated into cortical neuronal precursors for 20 days and mature neurons for 40 days in vitro (DIV). Precursors were characterized for key neuronal and cortical markers using SYBR green array, functional assays to measure lysosomal activity, and oxidative stress. **Results:** Genotyping of each frameshift mutation into exon 2 of the *SNCA* was confirmed by fragment analysis using capillary electrophoresis of fluorescently labeled PCR product. Immunocytochemistry of pluripotency test (*OCT4* and *SSEA4*) and SYBR green array for multilineage differentiation potential for each clone was confirmed with five genes per germ line. Bionano optical mapping resulted in normal karyotypes with no structural variance and with duplicated *SNCA* gene structure as the parental line. The absence of off-target binding sites of Cas9 is confirmed with Sanger sequencing. After the cortical neuron differentiation at 13 DIV, 16 DIV, and 20 DIV, we observed significant downregulation in general neuronal, cortical, and synaptic markers in the 4 KO line. Differentiated dopaminergic precursors in this model showed a reduction of oxidative and lysosomal activity in an (*SNCA*) gene-dose dependent manner. **Conclusion:** This isogenic *SNCA* gene dosage panel will be beneficial to address the physiological and detrimental effects of varying a-syn expression levels.

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536 Extru-seq: A Method for Predicting Genome-Wide Cas9 Off-Target Sites with Advantages of Both Cell-Based and *In Vitro* Approaches

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We present a novel genome-wide off-target prediction method named Extru-seq and compare it with cell-based (GUIDE-seq), in vitro (Digenome-seq), and in silico methods using promiscuous guide RNAs with large numbers of valid off-target sites. Extru-seq demonstrates a high validation rate and retention of information about the intracellular environment, both beneficial characteristics of cell-based methods. Extru-seq also shows a low miss rate and could easily be performed in clinically relevant cell types with little optimization, which are major positive features of the in vitro methods. In summary, Extru-seq shows beneficial features of cell-based and in vitro methods.

537 A Novel Method for Generating Unprecedented Ultra-High-Sequence Diversity Libraries of Plasmids and Recombinant Viruses (with Validation) for High-Throughput Screening and Targeted Therapy

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Background: A typical drug discovery pipeline needs good libraries of potential therapeutic candidates with high-sequence diversity. The probability of finding a good hit primarily depends on the quantity (diversity) and quality (distribution) of candidates in libraries used for screening. They include libraries of peptides, single-chain antibodies, nanobodies, CRISPRs, shRNAs, phage display, yeast display, Ribosome display, DARPins, BiKEs, TriKEs, CAR-T cells, and recombinant viruses (including adenoviruses, lentiviruses, AAVs, and other oncolytic viruses, etc.). Screening them for specific interaction with the target of interest to narrow down to fewer candidates, which can be further validated, are the basic steps in the generic discovery pipeline. ***Current limitations:*** Generating high-sequence diversity libraries is very challenging. Several published studies and industries claim to have very high diversity libraries ranging over 10^9 sequence diversity. However, most of those libraries lack validation by stringent experimental standards, and the diversity claimed is mainly based on sequencing a small number of candidates in the library and statistical extrapolations. Most of the time, we found that those who claim very high diversity (over 10^8) did not validate their libraries with stringent next-generation sequencing (NGS) based methods, and those who used stringent NGS methods for validation usually do not claim such big diversity. Therefore, there is an imminent need for a method to generate genuine high-sequence-diversity libraries of high quality. ***Methods and results:*** We have developed a novel method to generate such libraries with very high sequence diversity and validated them with stringent NGS-based methods. Compared to other established methods, our libraries are at least 100 to 1000 times better in diversity (quantity) and have a more uniform and unbiased distribution (quality). Through benchmarking studies, we validated total diversity, duplication rate, sequence bias, and the ability to scale up. As a proof of concept, we generated libraries of plasmids (shuttle vectors to make recombinant adenoviruses) and recombinant adenoviruses with high sequence diversity in a structural protein (Fiber). We also used them for screening against a cancer-specific cell-surface protein. ***Applications:*** Our methods can generate quantitatively and qualitatively better (100-1000 times) libraries. They can be used in high-throughput screening against targets of interest to narrow down to fewer candidates. Smaller libraries resulting after serial and multiple rounds of screening can serve as a dataset for machine training to aid AI-based design. Our unbiased, high-diversity libraries provide a high-quality dataset to improve Ai-based design criteria. We expect our libraries may help in avoiding AI-based design altogether in some cases.

538 An RNA Switch-Regulated All-in-One AAV-CRISPR-Cas System for *In Vivo* Inducible Gene Editing

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In vivo CRISPR-Cas gene editing has enormous potential for treating a range of human diseases. AAV vector is an efficient delivery vehicle for *in vivo* gene editing in diverse tissues. However, a single dose AAV delivered to terminally differentiated cells could provide persistent transgene expression for years to decades, which could lead to persistent off-target editing, long-lasting expression of an immunogenic Cas effector protein, and high level of double-strand break-associated integration of AAV vector DNA into chromosomal DNA. These potential safety issues significantly increase the risk and undercut the efficacy, and thus limit the application of this approach. An inducible CRISPR-Cas expression system has the potential to address these safety issues. We previously developed an RNA on switch system that efficiently regulates AAV transgene expression with up to 200-fold dynamic range in mice. In this system, a transgene is kept in off state by an optimized self-cleaving hammerhead ribozyme (T3H38), and can be conditionally switched on by a steric-blocking morpholino antisense oligonucleotide (v-M8) complementary to the ribozyme**.** Here, we utilized this efficient RNA switch to develop an inducible CRISPR/Cas system. We first tried directly introducing the T3H38 ribozyme to the 3′-UTR of a Cas9/Cas12a gene. Surprisingly, although the ribozyme efficiently lowered the expression of Cas9/Cas12a protein to almost undetectable level, gene-editing activity was much less impacted. More importantly, the difference between regulated and constitutive gene editing got smaller over time. This time-dependent leaky editing kinetics represent a key challenge for developing an inducible gene-

editing system. Then, by leveraging our previous finding that Cpf1/Cas12a can specifically bind and process its own CRISPR RNA from Pol-II transcripts, we engineered a four-level-regulated inducible CRISPR/Cas12a system that can be delivered by a single AAV vector. In this system, Cas12a protein expression, as well as CRISPR RNA expression, folding, and processing, are simultaneously regulated by the T3H38 ribozyme and a newly designed CRISPR-RNA-disrupting hairpin structure. We demonstrated in cell culture that, compared to a constitutive expression system, the four-level regulated system could function with 0% leaky gene editing activity in the absence of an inducer oligo and with up to 68% induced gene editing activity in the presence of a single v-M8 morpholino inducer. We then further tested the system in mice. Animals were continuously monitored for gene editing events for more than 6 weeks and minimal leaky editing activity was detected. The system was efficiently turned on when a single-dose (1.0 mg/kg) v-M8 morpholino inducer was administered. This RNA switch-regulated all-in-one AAV-CRISPR/Cas system has the potential to be used in diverse *in vivo* gene-editing applications.

539 De Novo Design of Peptide-Guided Protein Degraders with Structure-Agnostic Language Models

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Targeted protein degradation of pathogenic proteins represents a powerful new treatment strategy for multiple disease indications. Unfortunately, a sizable portion of these proteins are considered "undruggable" by standard small molecule-based approaches, including PROTACs and molecular glues, largely due to their disordered nature, instability, and lack of binding site accessibility. As a more modular, genetically-encoded strategy, designing functional protein-based degraders to undruggable targets presents a unique opportunity for therapeutic intervention. In this work, we integrate pre-trained language models with protein interaction databases to devise a unified, sequence-based framework to design peptide-guided degraders without structural information. We create a Structure-agnostic Language Transformer & Peptide Prioritization (SaLT&PepPr) module that efficiently down-selects peptides from known partner protein sequences for downstream screening. We experimentally fuse SaLT&PepPr-derived peptides to E3 ubiquitin ligase domains and reliably identify candidates exhibiting robust intracellular degradation of diverse pathogenic targets in human cells, including membrane proteins, disordered transcription factors, fusion oncoproteins, and other undruggable targets. We further show that our peptide-guided degraders have negligible off-target effects via whole-cell proteomics and demonstrate degradation of endogenous β-catenin and subsequent downregulation of Wnt signaling in cellular models of colorectal cancer. Finally, we encapsulate our peptide-guided degraders as mRNA in lipid nanoparticles (LNPs) and demonstrate effective in vivo delivery and target degradation in mouse models, motivating downstream clinical translation of our genetically-encoded degradation platform.

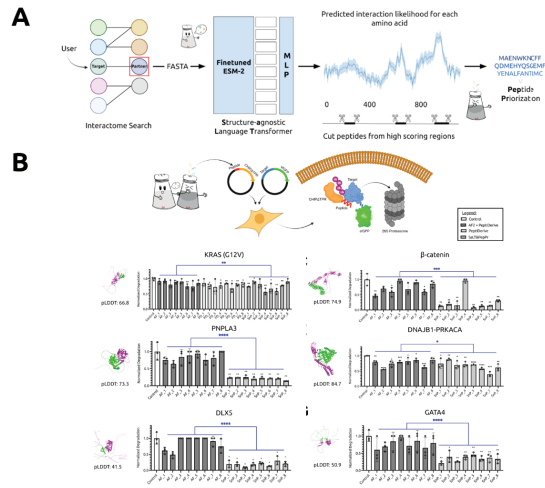

Figure. A) SaLT&PepPr model training and peptide prioritization pipeline. B) Testing of SaLT&PepPr-derived degraders vs. AlphaFold-derived degraders against diverse target substrates. AlphaFold structures are provided for each target with a pLDDT confidence score. All samples were conducted in independent triplicates (n=3). A two-tailed Student's *t* test was used to assess statistical significant degradation vs. a negative control for each sample, and a Mann-Whitney U Test was used to determine statistical significance between methods.

540 Efficient Circular RNA Engineering by End-to-End Self-Targeting and Splicing Reaction Using *Tetrahymena* Group I Intron and Study of Circular RNAs in Cells

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Circular RNA (circRNA) has advantages over linear mRNA which is gaining success as a new vaccine and therapeutic agent. However, new circRNA preparation method has not been developed for decades. In this study, we developed a new *in vitro* circular RNA engineering strategy by end-to-end self-targeting and splicing (STS) reaction during *in vitro* transcription using *Tetrahymena* group I intron ribozyme, providing circRNA without any extraneous fragments into circRNA sequences which may trigger unwanted innate immune responses in cells. For efficient self-circularization, we optimized group I intron ribozyme components and the optimized self-circularization RNA construct design showed the improved self-circularization efficiency which was comparable to permuted intron-exon (PIE) method. In addition, *in vitro* transcription condition for upstream process and Ion Pair-Reverse Phase HPLC (IP-RP HPLC) purification method for downstream process were optimized as well. IP-RP HPLC-purified circRNA with CVB3 IRES showed efficient protein expression in HEK293A cells comparable to circRNA prepared by PIE method. Moreover, circRNA without extraneous fragments and any modified nucleotides induced negligible innate immune responses in A549 cells. In conclusion, our new *in vitro* circRNA strategy efficiently generated circRNA *in vitro* for protein expression in cells and various applications.

541 Selection and Characterization of Truncated Vimentin Binding Aptamers

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Vimentin is an extracellular matrix protein expressed in normal mesenchymal cells to maintain cellular integrity. Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumors, central nervous system tumors, ovarian cancer, breast cancer, malignant melanoma, and lung cancer. Overexpression of vimentin has been associated with increased capacity for migration and invasion of the tumor cells. Recently, vimentin has been recognized as a marker for epithelial-mesenchymal transition (EMT). Based on its overexpression in cancer and its association with tumor growth and metastasis, vimentin also serves as an attractive potential target for cancer therapy. Aptamers are small single-stranded DNA or RNA ligands with high affinity and specificity to their targets. Aptamers offer many significant advantages over monoclonal antibodies in terms of low cost, non-immunogenicity, and facile modification for various applications. Along with nucleic acid design and synthesis techniques, aptamers have been rapidly developed for detecting and manipulating molecular changes, biomarker discovery, and targeted therapies. We have identified two phosphorothioated aptamers, V3 and V5, with high affinity and specificity binding to vimentin through our innovative morphology-based tissue aptamer selection (Morph-X-Select) method. Based on secondary structure prediction, we identified potential binding motifs of V3 and V5 by truncating the sequences on the stem-loop regions, and generated seven truncated motifs. Those tentative binding motifs were further characterized via cell and tissue binding assays, as well as flow cytometric analysis. By screening the binding affinity of the seven truncated motifs with vimentin-expressing IGROV cells, we selected V3M2 and V5M2 based on their highest fluorescence intensity among truncations of V3 and V5 groups, respectively. Truncations on the stem-loop regions demonstrated a significantly increased binding affinity (about 50% reduction of Kd values) to vimentin protein compared to that of the full-length V3 and V5 sequences while maintaining selective binding activity. Moreover, the shortened motifs with fewer thiophosphate modifications also led to reduced non-specific binding against both target and non-target proteins compared to the full-length phosphorothioated aptamers. Future applications for the vimentin binding aptamer motifs include conjugating the aptamers to synthetic dyes for targeted imaging, precise drug delivery, and targeted therapy in various epithelial cancers.

542 Multimodal Glioma Immunotherapy Combining TLR9-Targeted STAT3 Antisense Oligodeoxynucleotides with PD1-Specific Immune Checkpoint Blockade

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Introduction: Malignant gliomas (MG) are rapidly fatal despite multimodal treatments including radiation therapy, used to treat nearly all MG patients, or even the emerging cellular immunotherapies. Therapeutic resistance in glioma is related to tolerogenic STAT3 activity in both glioma cancer stem cells (GCSs) and in the tumor-associated myeloid immune cells, such as macrophages and microglia, which dominate MG microenvironment. We previously demonstrated that STAT3 activity in GSCs and tumor-associated myeloid cells can be targeted using Toll-like Receptor-9 (TLR9)-targeted oligonucleotide therapeutics such as siRNA or antisense oligonucleotides (ASO). **Methods:** Here, we describe development of a new TLR9-targeted and double-stranded STAT3 antisense oligonucleotide (CpG-STAT3dsASO) with optimized efficacy and safety for glioma immunotherapy. **Results:** Compared to our benchmark ASO oligonucleotides, the locked nucleic acid (LNA)-modified CpG-STAT3dsASO showed enhanced target gene knockdown in human and in mouse glioma cells and also in TLR9^{+} immune cells, such as macrophages and microglia. When tested against orthotopic model of human U251 glioma, intracranial injections of CpG-STAT3dsASO (1 mg/kg/q2w) inhibited tumor growth and significantly extended survival of immunodeficient NSG mice compared to benchmark oligonucleotide. Next, we tested CpG-STAT3dsASO against syngeneic GL261 and QPP8 models in immunocompetent mice. Our results demonstrated that CpG-STAT3dsASO was more effective and significantly better tolerated than single-stranded CpG-STAT3ASO when injected intracranially, without evidence of severe acute neural toxicities within tested dosing. All tested CpG-STAT3ASO variants induced activation of intratumoral DCs, macrophages and microglia, while reducing numbers of tumor-associated macrophages (TAMs), resting microglia and regulatory T cells as assessed using flow cytometry. To Our results also suggested the elevation of several immune checkpoint molecules on tumor-infiltrating T cells likely as a result of IFN signaling. Importantly, our preliminary experiments demonstrated a synergy between systemic PD1 inhibition with different dose (0.25 and 0.5 mg/kg) CpG-STAT3dsASO local therapy. While, neither of treatments alone was curative, the combination anti-PD1/CpG-STAT3dsASO therapy resulted in complete rejection of orthotopic GL261 tumors in the majority of treated mice. Importantly, CpG-STAT3ASO treatments improved the ratio of intratumoral CD8 T cells to Tregs. To elucidate changes in the glioma microenvironment related to STAT3-inhibition/TLR9-activation, we performed an initial single-cell RNAseq analysis of transcriptomic profiles in immune cell subsets isolated from tumors after treatment using CpG-STAT3dsASO^{LNA}. Our analysis indicated the reprogramming of tumor-associated myeloid cell populations

within treated glioma with an increased ratio of CD8:regulatory T cells. We previously demonstrated that GCSs can be targeted using TLR9/STAT3 antisense oligonucleotides. Here, our results indicated that the anti-PD1/CpG-STAT3dsASO treatment also inhibited tumor growth and extended the survival of murine GCSs-QPP8 model in immunocompetent mice. **Conclusions:** We believe that further development of CpG-STAT3dsASO will pave way to clinical translation of this strategy to immunotherapy of malignant glioma.

543 miR-375 Triggers Tissue Injury by Activating the Mitochondria of Macrophages

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Macrophage activation is central to human immune responses. MicroRNAs (miRNAs) are crucial regulators in various biological processes, including innate and adaptive immune responses. How miRNAs regulate macrophage activation is not well defined. Here, we found that lipopolysaccharide (LPS) stimulation increased miR-375 expression in multiple macrophage cell lines and mouse primary peritoneal macrophages. In an LPS-induced peritoneal inflammation mouse model, miR-375 expression was elevated in the duodenum, peritoneum, and liver. We isolated mouse primary peritoneal macrophages, infected with a lentiviral vector expressing miR-375 and reinfused into the peritoneal cavity of the recipient mice. Seven days later, recipient mice showed damaged duodenum and liver, and elevated levels of macrophages in tissues. Additionally, irregular duodenal villi, less hepatic lobules, granulocyte infiltration, and small areas of hemorrhaging were observed in the injured tissues. To investigate the impact of miR-375 on liver damage, we generated a liver-tropic scAAV9 vector expressing miR-375 under a ubiquitous cytomegalovirus enhancer/chicken β-actin (CMVen/CB) promoter and intravenously injected into adult C57BL/6 mice. Two weeks after injection, the mice showed increased aspartate transaminase (AST) and alanine transaminase (ALT) levels, and decreased body weights and liver sizes. Pathological analysis revealed severe inflammation and macrophage proliferation in the liver. In contrast, depletion of macrophages with clodronate liposome treatment suppressed the elevation of ALT/AST caused by the administration of scAAV9-miR-375 vectors. To explore the therapeutic applications, we generated an scAAV9 vector expressing an inhibitor of miR-375 and intraperitoneally injected it into adult C57BL/6 mice. Three weeks after injection, mice were challenged with LPS and sacrificed two days later. Enlarged spleens were observed in the mice treated with a control vector, but not with vector expressing the miR-375 inhibitor. Compared to control vector-treated mice, the mice that received vector expressing miR-375 inhibitor showed reduced cytokine (IFN-γ, IL-1β, IL-10, IL-17 and IP-10) and chemokine (MIP1β, MIP2 and MCP-1) levels in the serum. To understand the role of miR-375 on macrophage activation we established a stable mouse macrophage cell line that overexpresses miR-375. This cell model exhibited improved macrophage self-renewal, migration, phagocytosis, and activated mitochondria. Assessments of the metabolic capacity of the miR375 expressing macrophages using a Seahorse platform demonstrated a shift to glycolysis with a significative increase in the mitochondrial metabolic potential. This pattern is characteristic of M1 activation. The transcriptome analysis correlates with the upregulation of enzymes involved in glycolysis and in the mitochondrial biosynthesis of the building blocks for cytokines and itaconic acid. Finally, we found that miR-375 repressed *Zfp36l2* and upregulated WDR5 to stabilize cMYC to activate mitochondria. Altogether, this study revealed that miR-375 triggers tissue injury by activating the mitochondria of macrophages through a Zfp36l2-WDR5-cMYC axis. Using a miR-375 antagonist to prevent tissue injury caused by macrophage overactivation can be a possible therapeutic approach. # J.H. and Y.W. are co-first authors *N.H., G.G., and J.X are co-corresponding authors

544 Self-Delivering RNAi (INTASYL™) Compounds Targeting TIGIT or CBL-B Improve NK Cell Functional Cytotoxicity toward Tumor Cells

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Background: NK cells are the body's first line of defense against cancer, able to recognize and kill tumor cells without prior exposure. Unlike T cells, allogeneic NK cells do not induce graft-versus-host disease. NK cell adoptive cell therapy (NK-ACT) holds promise as a true "off-the-shelf" cellular immunotherapy for cancer, with the potential to circumvent many of the hurdles associated with autologous cell therapies. NK-ACT shows promise against hematological cancers, but the cytotoxic activity of these cells needs to be enhanced to improve clinical efficacy. In particular, expression of inhibitory proteins such as TIGIT and CBL-B has been shown to inhibit NK cell-mediated cytotoxicity. Therefore, strategies to relieve TIGIT and/or CBL-B inhibition in NK-ACT products are under active exploration as means to improve the efficacy of NK-ACT. Incorporating RNAi treatment into ex vivo NK cell expansion protocols prior to ACT represents one such strategy. The INTASYL™ platform is a self-delivering RNAi technology that imparts small molecule-like properties to interfering RNAs, providing extremely efficient delivery to target cells without need for specialized formulations or drug delivery systems. This allows INTASYL to be easily incorporated into cell therapy manufacturing protocols. INTASYL compounds are potent, stable, and are rapidly taken up by cells. While durable, INTASYL-mediated gene silencing is transient, mitigating safety considerations that may arise from permanent deletion of NK cell suppressors. INTASYL compounds are able to silence both extracellular (TIGIT) and intracellular (CBL-B) proteins. **Methods:** Primary human $CD56^+$ NK cells were expanded using the ImmunoCult™ NK Cell Expansion Kit. Following the 14-day expansion protocol, cells were collected, and the cell density was adjusted to 0.5 x 10^6 cells/mL in culture media containing IL-2. Cells were seeded directly into 24-well plates containing INTASYL compounds ranging in final concentration from 0 to 5 μM. TaqMan gene expression assays were used to determine expression levels of TIGIT and CBL-B following the RNA-to-Ct 1-step protocol. Cytotoxic capabilities of the INTASYL-treated NK cells against the K562 (Chronic Myelogenous Leukemia) cancer cell line were tested in a DELFIA cell

cytotoxicity assay. Following co-culture, cell culture supernatants were analyzed by ELISA for increased secretion of IFN-γ. **Results:** INTASYL treatment results in consistent mRNA silencing without negative impact on NK cell viability. Silencing of TIGIT and CBL-B resulted in increased expression of markers of NK cell activation and increased cytotoxic capabilities of NK cells against K562 cancer cells in the DELFIA cell cytotoxicity assay. **Conclusions:** Here, we demonstrate the potential of INTASYL to improve NK cell potency in ACT. By treating NK cells with INTASYL targeting the inhibitory proteins TIGIT and CBL-B ex vivo, during NK cell expansion, the anti-tumor response of these cells was enhanced. This may result in a more effective cell therapy for solid tumors and hematological malignancies. In vivo studies are ongoing.

545 Cell-Selective STAT3 PROTAC Degrader Based on the Decoy Oligonucleotide

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Oncogenic and/or tolerogenic transcription factors (TFs), such as STAT3 and NF- kB, are enticing but challenging targets for cancer therapy. Decoy oligodeoxynucleotides (dODNs) specific to TF DNA-binding domains were explored as highly-selective and competitive TF inhibitors. However, delivery challenges and limited potency hampered translation of decoy inhibitors. We previously demonstrated that conjugation of dODNs to single-stranded CpG ODNs facilitates targeted delivery to cells expressing scavenger receptors (SRs)/Toll-like receptor 9 (TLR9), such as myeloid cells and certain cancer cells. To improve inhibitory potency of dODNs, we converted a STAT3-specific decoy (S3dODN) into a proteolysis targeting chimera (S3dODN^{PROTAC}) capable of inducing an irreversible STAT3 protein degradation. Thalidomide molecule (Tha), a ligand for cereblon (CRBN) within a E3 ubiquitin ligase complex, was tethered to the 3' end of S3dODN. The S3dODN^{PROTAC} design was guided by computational modeling in solution to enable complexing of STAT3 proteins bound to S3dODN^{PROTAC} via CRBN to the E3 ubiquitin ligase complex. STAT3 protein degradation and decreased activity was almost completely abrogated in target myeloid cells transfected with STAT3dODN^{PROTAC} but not with equimolar amounts of unconjugated S3dODN and Tha. The competition studies verified specific contributions of decoy and thalidomide moieties in STAT3 degradation. The inhibitory effect of S3dODN^{PROTAC} in target cells was abolished in the presence of proteasome inhibitors and also by CRISPR-knockout of CRBN consistently with the putative proteolytic mechanism of STAT3 inhibition. Based on modeling studies, we have identified two lysine residues, K601 and K626 as potential ubiquitination sites in STAT3. The mutation of either site or both sites together reduced or completely abrogated S3dODN^{PROTAC}-induced STAT3 degradation, respectively. To enable targeted and formulation-free STAT3dODN^{PROTAC} delivery, we conjugated this ODN to a CpG moiety. C-S3dODN^{PROTAC} was spontaneously internalized and reduced STAT3 levels in primary mouse, myeloid cells, B cells and human Ly18 and mouse A20 B cell lymphoma cells, but not in T cells. C-S3dODN^{PROTAC}, but not the unmodified C-S3dODN, decreased STAT3 levels to 50% at 250 nM and over 85% at 2 μM dosing in mouse myeloid cells. The inhibitory effect of C-S3dODN^{PROTAC} was specific to STAT3 and it did not affect levels of the related STAT5 and STAT1 proteins. To our knowledge, this is the first demonstration that the PROTAC approach can be successfully applied to decoy-based targeting of undruggable transcription factors such as STAT3.

546 Development of a Next-Generation *Ex Vivo* Delivery Platform Using Nanoneedles for Physical Cargo Delivery into Cells

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The widespread use and development of cell therapies has been limited by a deficiency of efficient and safe delivery methods. The current conventional methods of biomolecule delivery all have severe restrictions, including a lack of dose control, a lack of precision, patient safety concerns, cell viability issues, cargo size limitations, and cell type restrictions. All these limitations compound and can cause both short- and long-term cell functionality compromises that are observed either in the production of a cell therapy or downstream in the patient. At Mekonos, we are developing a next-generation delivery platform that will address the *ex vivo* delivery bottleneck and unlock the full potential of gene editing for cell therapies. Here we describe our novel cell engineering platform which utilizes a controllable array of solid silicon nanoneedles to enable direct intracellular access of multiplexed biomolecular payloads without the use of viral vectors or electroporation. The silicon nanoneedles are made using an advanced semiconductor process to develop chips that are scalable and precise, allowing us to consistently produce nanoneedles with specific heights and widths. Cells are trapped using a microfluidic system that enables precise localization of each nanoneedle tip to a single cell, enabling a controlled, consistent delivery that is not possible with other delivery methods such as electroporation. The biomolecular payloads are adsorbed onto the surface of each nanoneedle with an aptamer-based surface chemistry that ensures the controlled release of the desired biomolecule once within a cell-specific environment. Our nanoneedle delivery method has been demonstrated to be cell type and cargo agnostic, allowing us to deliver a variety of biomolecules such as nucleic acids, proteins, and RNP complexes into a variety of primary and stem cells with higher viability and efficiency than current industry standard methods. We believe that the combination of our semiconductor, microfluidic, and surface chemistry technologies will drive the future of cell therapies through scalable, hyper-precise cell engineering.

547 A GMP-Compatible Process for the Efficient Transfection of MSCS with mRNA

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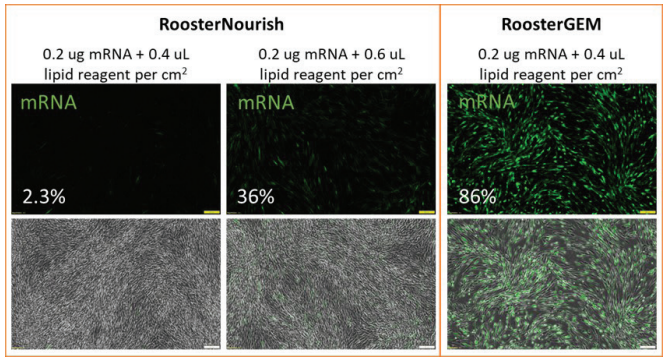
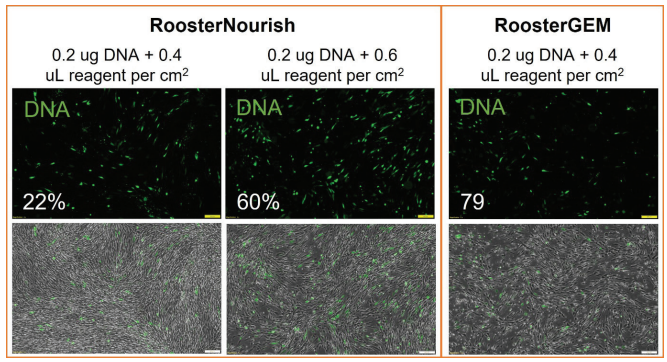
Background & Aim Genetic engineering of mesenchymal stromal cells (MSCs) has emerged as a powerful player in the cell and gene therapy field due to MSCs' immunomodulatory and regenerative potential. For example, transient transfection of MSCs has been shown to enhance neovascularization *in vivo* via increased MSC survival. mRNA transfection is well suited for cellular reprogramming (i.e. gene editing) applications, as a transposase or Cas9 need only be expressed while integrating a gene of interest. We previously optimized medium formulations and mRNA transfection parameters for MSCs and demonstrated dramatic enhancement of MSC transfection efficiency. A significant challenge to the clinical implementation of mRNA-modified MSCs is to efficiently transfect MSCs within the context of GMP-compatible processes. Therefore, we aimed to develop a scalable GMP-compatible process for genetically modified MSCs that could be rapidly implemented within product development programs. Methods, Results, & Conclusion MSCs were plated in 2D flask culture for 4 days until 90% confluent and passaged into a final culture vessel using RoosterNourish medium. After 3 days, the growth medium was changed to RoosterGEM (Genetic Engineering Medium) formulated with all-GMP reagents, and MSCs were transfected using a proprietary transfection reagent developed by Mirus Bio and mRNA or plasmid DNA encoding a fluorescent protein. Cell transfection efficiency was evaluated using fluorescence microscopy and quantified via flow cytometry. The transfection reagent yielded 86% mRNA transfection efficiency and 79% DNA transfection efficiency (**Figures 1, 2**). Despite an initial overall lower cell yield in RoosterGEM compared to RoosterNourish growth medium, there were still fifteen-fold more engineered cells in the RoosterGEM process when transfected with mRNA. To ensure this process yields a viable product, we showed that mRNA-transfected MSCs can be cryopreserved and thawed successfully, maintaining their protein expression (**Figure 3**). Cells lose their fluorescence rapidly after passage, with only 20% of cells maintaining fluorescence after 3 days. Therefore, we recommend transfecting cells at the confluence and passage desired for the final application. We have also tested MSC expansion and transfection in a 3D bioreactor process, with transfection efficiency of ~50% using RoosterGEM using un-optimized conditions. Future work will optimize the transfection of MSCs with mRNA in a 3D bioreactor process.





548 Epicardial Hydrogel Delivery of Adeno-Associated Virus to Treat Heart Disease

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Introduction: Heart disease is a leading cause of death world-wide. Despite promising preclinical studies, clinical trials using adeno-associated virus (AAV) to treat heart disease have failed due to low transduction of cardiomyocytes (<1%) and neutralizing effects of anti-AAV antibodies. Hydrogels containing non-viral therapeutics delivered to the epicardium through the pericardial space have shown efficient targeting of drugs to the heart while minimizing off-target delivery. The goal of this study is to investigate the delivery of AAV-containing hydrogels to the epicardium. We hypothesize that epicardial hydrogels encapsulating AAV will efficiently transduce cardiomyocytes *in vivo*. **Methods:** AAV9 delivering mCherry driven by the CMV promoter was encapsulated in 4-arm poly(ethylene glycol) (PEG-4MAL) hydrogels crosslinked with protease-sensitive peptide and hydrolytic crosslinker. AAV was delivered to male Sprague-Dawley rats at 2.5E11 vector genomes (vg)/rat by 50 µL epicardial gels (n=4). Control (no AAV) epicardial gels were also included (n=4). Eight animals were randomized to receive either control gel or AAV gel. Organs were collected for analysis 6 days post-treatment. Transgene expression in cardiac tissue was analyzed by quantitative PCR (qPCR) and immunohistochemistry. Masson trichrome and H&E staining were used to evaluate potential inflammation or fibrosis induced by AAV. **Results:** By qPCR, we measured fold changes in mCherry expression in the heart relative to a reference gene (PPIA) of 0.076 ± 0.050 in animals that received epicardial AAV gels (n=4). No amplification was detectable in control animals (n=4). Immunohistochemical analysis of the heart tissues from epicardial AAV gels showed mCherry expression across the epicardium and myocardium. Signal colocalized with cardiac troponin T (cTnT) as a cardiomyocyte marker. Histological staining did not show evidence of inflammation or fibrosis, and there were no significant increases in fibrosis accumulation measured by colorimetric analysis using ImageJ in heart tissues from AAV gels compared to

control gels. **Conclusion:** These results provide a basis for AAV-containing hydrogel delivery to the epicardial surface. Future work will focus on optimizing the hydrogel for AAV cargo and evaluating local and systemic immunologic response to epicardial delivery.

549 New Imidazole Core Based Helper Lipids for Non-Viral Gene Delivery Applications

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Introduction Development of efficient, reinjectable and non-toxic gene delivery systems is among the most challenging requirement to deploy the full potential of gene therapy. As an alternative to viral-based systems that are associated with immunogenic and oncogenic complications, synthetic carriers have been developed, notably based on cationic lipids and polymers. In this context, we have designed and synthesized four new helper lipids (HpLs) featuring either one or two identical imidazole or histidine moieties as polar region. These moieties can be protonated in acidic environment, destabilizing endosome membrane for an easier endosomal escape induced by causing swelling and rupture of the endosomal membranes. In this work, our goal was to improve non-viral gene transfection by associating BSV107, a cationic lipid (CL), with novel HpLs and to compare them with formulations including DOPE. To better understand the impacts of imidazole rings during gene delivery, we further studied the endosomal escape pathway implicated during transfection. **Methods** Formulations with cationic lipid BSV107 and HpLs were prepared at ratio 1:1 and their physicochemical parameters (size, zeta potential and polydispersity index) were determined by dynamic light scattering. Transfection efficiencies (expressed in relative light unit per mg of total proteins, RLU/mg) were determined using a plasmid encoding a luciferase reporter gene 24h after transfection and cell viabilities were also evaluated. Transfection assays were performed mainly on human epithelial lung cell lines with different charge ratios (2, 4 and 6) between CLs and plasmids. To explore the endosomal escape, the impact of the new HpLs was probed by using different endosome maturation inhibitors which were added to the medium two hours prior to transfection assays. **Results** Formulations including HpLs demonstrated a significantly higher transfection efficiency compared to standard CLs in the three cell lines used (10^6 RLU/mg for CLs vs. 10^7 - 10^8 for HpLs-CLs). In term of transfection efficiencies, no significant difference was observed between histidine and imidazole-based HpLs. HpLs containing two moieties (either imidazole or histidine) showed better transfection efficiencies than HpLs with only one moiety (10^7 vs 10^8 RLU/mg). Experiments performed with two rings-based HpLs-CLs demonstrated transfection efficiencies (10^8 RLU/mg) and cell viabilities (70% of untreated cells) comparable with DOPE-associated CLs. However, we noticed higher toxicities (approximately 20%) with histidine-based HpLs-CLs. In addition, the application of endosome acidification inhibitors (bafilomycin A1, a specific inhibitor of vacuolar-type H+ ATPase, for example) reduced significantly the transfection efficiency of HpLs-CLs complexes. These results highlighted the importance of acidic endosomal pH in transfection processes. **Conclusion** In this work, the synthetic vector BSV107, associated with our innovative imidazole/histidine-based HpLs, have demonstrated promising transfection efficiencies. Further optimizations are still needed to enhance biocompatibility and reduce potential toxicity. In this sense, the application of microfluidic should result in safer formulations. The role of endosomal pathway could be further characterized through cellular sub-fractionation and microscopic studies. This will help us to better understand various steps of transfection processes and to fine-tune our formulations to fit with future applications.

550 Development of Blood-Brain Barrier-Penetrating Functional Lipid Nanoparticles for Effective CNS Gene Therapy

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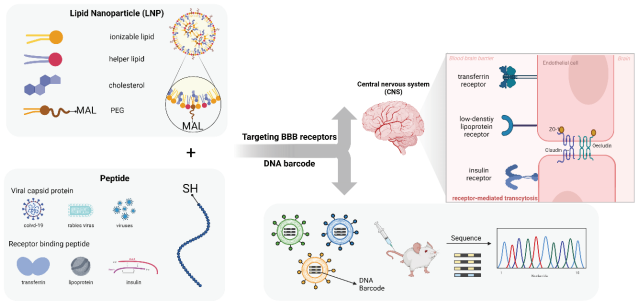

Despite many current treatment options that could treat central nervous system (CNS) diseases, there still remains a need to improve the delivery efficacy of drugs into the brain. This is because there is a blood-brain barrier (BBB) that selectively passes nutrients from the blood to maintain brain neuron function and control the brain tissue's microenvironment. Approaches to penetrating the BBB to deliver LNP into CNS for gene therapy are being conducted, but the problem is that the effectiveness is still low. In this study, we formulated LNPs capable of crossing the BBB by tailoring the composition and surface properties of LNPs to promote their binding to receptors that are present in the BBB and inducing receptor-mediated transcytosis. Furthermore, it is generally believed that the smaller size is more efficient to cross the BBB. Therefore, for effective CNS gene therapy, we prepared sub-100nm LNPs and functionally modified the particle surface with peptides capable of targeting the receptor in the BBB. This approach involves incorporating functional peptides selected from diverse proteins, including viral capsid proteins, known to efficiently penetrate the BBB. In this presentation, diverse LNP formulations with functional peptides for improving the efficacy of CNS gene therapy will be compared and discussed in more detail.

551 Demonstration of Safety and Therapeutic Effect of Cell-Derived Vesicles in Retinal Degeneration

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Introduction: Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries. Despite the emergence of ophthalmic drugs, effective treatment for AMD remains elusive. It was widely reported that inflammation is the key to AMD progression. Previously, we have reported the immunomodulatory capacity of cell-derived vesicles (CDVs) in several inflammatory or degenerative diseases. Here, we present the evidence to support the potential of CDVs as a novel therapeutic for slowing the progression of AMD. The safety and therapeutic potential of CDVs were assessed by examining retinal function in an AMD mouse model. **Methods**: CDVs produced from umbilical cord mesenchymal stem cells (UCMSCs) by serial extrusion were used in this study. RNAseq was first performed to identify key miRNAs involved in anti-inflammatory functions in UCMSC-CDVs. Then, the cellular uptake of CDVs in different retinal cell types was assessed *in vitro*. *In vivo* safety and efficacy of UCMSC-CDVs were evaluated after administering CDVs by intravitreal injection into mice subjected to photo-oxidative damage for 5 days. Alteration in retinal function was measured using electroretinography (ERG), angiography, and immunohistochemistry. **Results**: Comprehensive bioinformatics analyses of RNAseq data revealed that UCMSC-CDVs were enriched with let-7 family and miR-125a, well known for their therapeutic protection in retinal degeneration. CDVs showed efficient cellular uptake in different retinal cell types. In the *in vivo* experiments, UCMSC-CDVs demonstrated a protective effect against retinal degeneration, while exhibiting no apparent toxicity in the mouse retina. Overall, UCMSC-CDVs significantly improved retinal function and reduced levels of cell death as measured by the number of photoreceptor rows and $TUNEL^+$ cells in the outer nuclear layer. Additionally, treatment of UCMSC-CDVs did not induce inflammation in the retina. **Summary/Conclusion**: Findings of this study support the use of UCMSC-CDVs as non-toxic and therapeutically efficacious vesicles in retinal degeneration. With proven productivity and versatility for various drug loading, UCMSC-CDVs can serve as effective therapeutics as well as vehicles for additional payloads in AMD and many other inflammatory diseases.

552 Development of Highly Efficient and Biocompatible Delivery Method of RNA Therapeutics Using BioDrone™ Platform Technology

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Introduction: The extraordinary success of CoVid-19 vaccine has accelerated an explosive expansion of mRNA therapeutics. However, synthetic vehicles such as lipid nanoparticles (LNPs) and polymers currently used for mRNA delivery exhibited considerable safety concerns. Cell-derived vesicles (CDVs) produced by serial extrusion of diverse human cells are emerging as novel delivery vehicles for RNA delivery due to their superior biocompatibility, capability to cross diverse tissue barriers, and unique scalability. Here, we present an alternative approach for mRNA delivery using CDVs to overcome the immunogenicity and toxicity issues. The therapeutic potential of CDVs carrying mRNA was evaluated *in vitro* and *in vivo*. **Methods**: mRNAs were complexed with CDVs via positively-charged lipids. Fluorescent-labeled mRNAs or reporter mRNAs such as luciferase and EGFP were used to determine the potential of CDVs for mRNA delivery. Cellular mRNA uptake and protein expression level were measured using flow cytometry, western blot, and luciferase reporter assay. The *in vivo* delivery and biodistribution of mRNA-loaded CDVs were assessed by IVIS imaging. In addition, acute toxicity and inflammatory response were evaluated by assessing a hematology profile and multiplex cytokine/chemokine panel. All the animal experiments were conducted in comparison with LNPs. **Results**: We established a method of loading mRNAs to CDVs using cationic lipids. The mRNA cargo can be loaded onto CDVs with over 90% of loading efficiency, maintaining the integrity of the vesicle structure. CDVs facilitated the delivery of mRNAs to target cells, resulting in robust protein expression. Remarkably, no significant immunogenicity was observed upon intramuscular and intravenous delivery of mRNAs by CDVs, whereas multiple immunogenic responses were evident in LNPs. **Summary/Conclusion**: Taken together, we have demonstrated the potential of CDVs as an mRNA delivery carrier. With proven safety and efficiency, the BioDrone™ technology based on CDVs will expedite the development of mRNA-based therapeutics for cancer, CNS disorders as well as many other debilitating diseases.

553 A Novel Phosphatidylserine-Based Nanoparticle Gene Delivery System for DNA & mRNA

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Background: A major challenge confronting modern-day gene therapy is the safe, efficient, and targeted intracellular delivery of DNA and mRNA. Currently available options include lipid nanoparticles (LNPs) and viral vectors, both of which have a number of well-documented limitations. We wish to report recent results using a novel phosphatidylserine (PS) based nanoparticle (PS-NP) gene delivery platform to deliver plasmid DNA and mRNA in vitro. **Methods:** Studies were conducted in cultured HeLa cells. Cells were transfected with 200 ug/mL of either GFP or luciferase-coding plasmid DNA or

mRNA (Tri-link) that was encapsulated in a PS-NP delivery platform. In vitro cell culture doses ranged from 4-500 ng per well, with controls of the respective nucleic acids delivered either by Lipofectamine 3000 or Lipofectamine™ MessengerMAX™Transfection efficiency was assessed by fluorescence microscopy and flow cytometry, looking at both fluorescence intensity and the percentage of GFP-positive live cells. Cytotoxicity was assessed by cell viability with ATP Glo assay. Stability of mRNA formulations was evaluated by measuring the transfection activity of the PS-NP mRNA formulations at time 0, and after 1,2,3, 5, and 10 weeks of storage at 4 ºC storage conditions. **Results:** For each of the PS-NP/oligo formulations studied, particle sizes ranged from 100 to 400 nm, with Zeta potentials from 30 to 60 mV. Efficiency - pDNA formulations were as potent as Lipofectamine 3000 controls at concentrations ranging from 4-500 ng. mRNA formulations were less active than gold-standard Lipofectamine™ MessengerMAX, but still showed substantial clinically relevant activity.Cytotoxicity testing showed virtually no cell death after 36 hours of incubation at high doses of 500 ng DNA per well, with similar survival with sequential serial dilutions down to 31 ng.Stability (and biological activity) of mRNA formulations was maintained out to 10 weeks. **Conclusions:** This unique PS-NP delivery platform represents a promising delivery alternative for gene therapy applications that require safe, efficient delivery of DNA and mRNA. Confirmatory in vivo work is ongoing.

554 Lipid Nanoparticle-Mediated Gene Editing of Human Primary T Cells and Off-Target Analysis of the CRISPR-Cas9 Indels

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Background and Aims: TheCRISPR/Cas9 system has emerged as a powerful tool for gene editing of primarycells. In our previous work, we demonstrated a novel lipid nanoparticle (LNP)reagent for the multi-step engineering of gene-edited CAR T cells. We showedhigh cell viabilities and potent CAR-T mediated killing, even after multiplegenetic manipulations. Here, we extend this work by assessing potentialoff-target editing effects in both LNP-treated T cells and T-cells where theCRISPR reagents were delivered by electroporation. Further, we evaluatedmultiple Cas9 variants and guide RNA targets. **Methods**: LNPsencapsulating wild type or high fidelity S.p. Cas9 mRNA and various *TRAC*and *CD52* targeted guide RNAs (sgRNAs) were produced using our scalable NanoAssemblr™ microfluidicsplatform. Concurrently,electroporation was performed to deliver equivalent cargoes. Purified primary Tcells were cultured, activated, and expanded in serum-free media in plates,flasks, or small bioreactors. The LNPs were added to cells by direct additionfor gene editing. Gene expression and cell viability were measured using flowcytometry or colorimetric assays. Multi-target performance of CRISPR-Cas9editing was evaluated through rhAmpSeq™ next-generation sequencing (NGS) and indels analyzed for with CRISPRAltRations.**Results**: TCR or CD52 targeted Cas9 mRNA-LNPaddition or electroporation yielded high single and double knockoutefficiencies. High-throughput NGS analysis showed strong agreeance to flowcytometry for on-target analysis. We tested a range of sgRNA targets, wild-typeand high-fidelity Cas9 mRNAs, and determined off-target editing for all targetsand variants investigated. Similar results were obtained when comparingdifferent LNP batch sizes (micro to milligram RNA) and cell culture vessels(0.1 to 45 million cells), demonstrating scalability of both LNP production andcell treatment.**Conclusions**: The results from this study further supportthe utility RNA-LNPs for the genetic engineering of primary T cells. The simpleand gentle nature of LNP cell treatment allows for multiple genetic engineeringsteps for simultaneous expression and deletion of proteins. These LNPs can be easily manufactured fromsmall scale to rapid scale up.

555 False Positive Gene Editing Outcomes as Measured by Protein Expression Following Polymer-Based CRISPR Delivery Due to Nonspecific Sequestration of Antibodies by PEI

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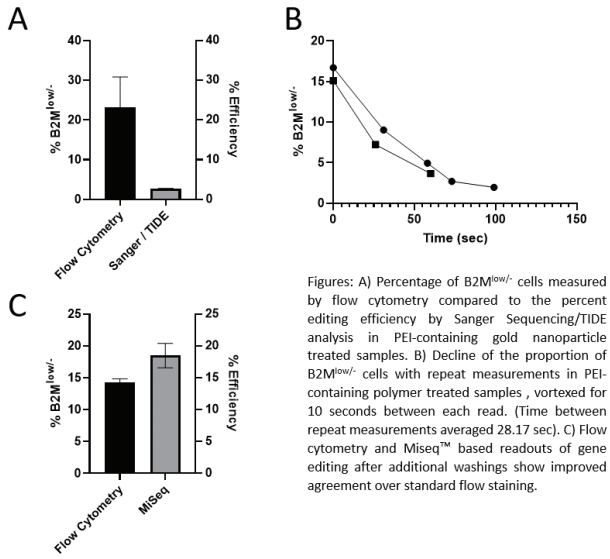
CRISPR gene editing can create insertions and deletions (indels) in genomic DNA to alter protein expression in cells for therapeutic purposes. Current state of the art uses electroporation to deliver CRISPR plasmids or ribonucleoprotein (RNP) complexes, however these methods are complex and expensive to implement, and not easily translated *in vivo*. There are several major classes of nanoscale delivery tools being developed to enable *in vivo* CRISPR therapy base on: cationic lipids, polymers, and/or metal cores, including gold (AuNP). Both polymeric and metal core nanoparticles utilize cationic polymers as oligonucleotide carriers and intracellular release agents. Transgenic murine models have been used to evaluate biodistribution and activity of these *in vivo* CRISPR delivery tools, however, these models are not reproducible across delivery platforms/tissues. An ideal model would enable rapid and reliable readout of gene editing frequency and biodistribution, preferably as changes in functional protein expression. Here we studied the four exon human β-2-microglobulin (B2M) gene as a target for comparing CRISPR delivery strategies. As a component of the major histocompatibility I complex, the B2M protein is expressed on the surface of most tissues and cell types. Indels in the coding sequence result in loss of B2M expression, making flow cytometry a useful, rapid readout for gene editing. As proof for B2M's utility we evaluated a cationic, polyethylenimine (PEI)-containing AuNP for its ability to deliver CRISPR (Cas9) RNP into Jurkat cells. Comparing untreated Jurkat cells and electroporation controls, cells were stained for flow cytometry on a BD FACSCelesta and analyzed on FlowJo™ software (v.10.8). Gating first identified cells using forward and side light scatter, then gated on live cells (Zombie NIR™) followed by B2M Mouse anti-human, Clone: 2M2. We observed 23.13±13.34% $B2M^{low/-}$ cells across all AuNP + PEI treated samples. However, Sanger sequencing with TIDE analysis showed 2.67±0.21% gene editing, suggesting flow cytometry results were false positive. We hypothesized that PEI could interfere with antibody staining due to known electrostatic PEI-protein interactions leading to sequestration.

To test this, we repeated flow cytometry against a PEI-containing polymer and tracked its change in B2M protein expression. Repeat measurements of the same samples demonstrated an apparent decrease in proportion of B2M$^{low/-}$ cells over 5 min from 16.70% to 1.96% with repeated mixings. To fix this problem, we developed a new staining protocol with maximum volume dilution and additional washing steps to remove excess PEI prior to staining for B2M. Consequently, we observed ratiometrically stable frequencies of gene editing by both flow cytometry (14.23±1.04%) and MiSeq™ analysis (18.50±3.30%), confirming that presence of PEI could lead to false positive gene editing results. This study underscores the need to optimize analysis protocols for protein expression in intact cells when PEI-containing polymers are used for delivery of gene editing tools, and to validate gene editing outcomes by multiple methods at different points in the central dogma of biology.



Figures: A) Percentage of B2M$^{low/-}$ cells measured by flow cytometry compared to the percent editing efficiency by Sanger Sequencing/TIDE analysis in PEI-containing gold nanoparticle treated samples. B) Decline of the proportion of B2M$^{low/-}$ cells with repeat measurements in PEI-containing polymer treated samples , vortexed for 10 seconds between each read. (Time between repeat measurements averaged 28.17 sec). C) Flow cytometry and Miseq™ based readouts of gene editing after additional washings show improved agreement over standard flow staining.

556 High-Throughput Screening Platform of Lipid Nanoparticles for *In Vivo* CRISPR/Cas Gene Editing

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Gene editing mediated by CRISPR/Cas holds great promise for gene therapy. Delivery of CRISPR/Cas systems to specific tissue by an effective way is one of the greatest challenges in *in vivo* gene editing. Lipid nanoparticle (LNP)-based mRNA delivery represents a clinically proven and effective nonviral delivery system that is gaining attractions across the fields of the nucleic acid-based therapies. Previously, thousands of novel lipid components and LNP compositions were screened either in cell culture contexts or *in vivo* to evaluate single mRNA delivery and expression. However, these screening strategies may not be applicable on CRISPR/*Cas9* mRNA-based *in vivo* gene editing, which needs codelivery of both *Cas9* mRNA and target single-guide RNA into one cell and function as one ribonucleoprotein complex. A high-throughput platform for screening LNPs dedicated to the *in vivo* delivery of functional CRISPR/Cas9 systems is in great demand. We herein present an *in vivo* high-throughput screening platform for simultaneously quantifying delivery efficiencies of up to 96 barcoded LNPs encapsulating *Cas9* mRNA and single-guide RNA (sgRNA) by single administration with pooled LNP mixture and subsequent deep sequencing. In the initial proof-of-concept experiments, we designed an array of LNPs consisting of combinations of new lipids, formulations, and encapsulation processes. The delivery efficiencies of different LNPs were tracked and quantified by their corresponding barcodes, in which the sequence *per se* has no effect on delivery and can only be detected when both *Cas9* mRNA and target sgRNA are co-delivered and function within cells. In the context of *in vivo* delivery to rat liver, several barcodes showed high enrichments, indicating that the corresponding LNPs exhibited high delivery efficiencies for CRISPR/Cas9. Indeed, the results were subsequently verified by individual LNP delivery in individual rats, indicating that this platform can efficiently identify lead LNP candidates as well as optimal formulation parameters for *in vivo* delivery of CRISPR/Cas9. We further applied this approach to nonhuman primate (cynomolgus monkey) and screened for 96 LNPs. By measuring the relative abundances of the 96 LNP barcodes, we identified new formulations and lipids that were associated with varying delivery efficiencies of CRISPR/Cas9. Interestingly, those LNPs showed high delivery and editing efficiencies in cynomolgus monkey were not always the same as those showed high efficiencies in rats, and vice versa. The result suggests that the efficiency of LNP-based mRNA delivery of CRISPR/Cas9 system may differ across different species, highlighting the significance of choosing proper *in vivo* LNP screening models for LNP-based gene editing therapies. Our screening platform employs a direct quantification of barcoded LNP harboring functional CRISPR/Cas9, enables a high-throughput and straightforward readouts of efficient LNP-*Cas9* mRNA delivery systems. The high-throughput platform could be broadly applied beyond LNPs to non-lipid nanoparticles and beyond Cas9 nucleases, thus would significantly benefit *in vivo* CRISPR/Cas therapeutic applications.

557 Edible Plant-Derived Nanovesicles to Systemic Deliver Nucleic Acid Medicine for Oral Administration

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[Introduction] In recent years, as the functions and properties of extracellular vesicles, including exosomes, have been elucidated, the development of drug delivery systems (DDS) based on these properties has been actively conducted. On the other hand, several issues remain to be cleared in supplying extracellular vesicles for clinical application, including cost, quality, and storage. Therefore, we searched for exosomes that could be mass-produced at low cost, and found that exosome-like vesicles could be extracted from acerola juice with high efficiency. The aim of this study is to deliver siRNA of ApoB, a major protein component of Low Density Lipoprotein (LDL), to the target tissue (liver) by oral administration using plant-derived nanovesicles as DDS carriers. [Methods] Acerola juices were obtained from Nichirei Foods Inc. The exosome-like nanovesicles derived from acerola juices (AELNs) were extracted by exoEasy Maxi kit (Qiagen). Nanoparticle tracking

analysis (NTA) measurements were performed using Nanosight LM10 system (Malvern). ALENs (1×10^9 particles/ml) and ApoB siRNA (100 pmol, Ajinomoto Bio-Pharma) were mixed in 50 µl of PBS. After incubation on ice for 30 min. were used to measure the oral administration of ApoB siRNA. Oral administration was conducted with total volume 200 µL sample (including 10mg/kg of synthetic ApoB siRNA-AELN complex) by the sonde. The C57BL/6 female mice (8 weeks old, n = 5 per cohort) treated with synthetic ApoB siRNA-AELN complex were scarified and each organ was collected at 1h or 24 h after oral administration, respectively. The expression of ApoB gene in each organ were measure by qRT-PCR. [Results] The number of exosome-like nanovesicles in 8 ml of lemon juice was found to be 1×10^8 particles, whereas acerola juice contained 1×10^{12} particles of AELNs, a larger amount than other fruits. Thus, acerola was shown to be an excellent source of exosome-like nanovesicles.We found that it is possible to form a complex by mixing AELNs (2.2×10^9 particles) and synthetic miRNA (100 nM) at a certain concentration and allowing them to stand on ice for 30 minutes. Furthermore, the nucleic acid (synthetic miRNA) that formed a complex with AELNs was resistant to RNase, strong acid (pH 2), and strong alkali (pH 10). The in vivo system was used to analyze the in vivo kinetics of acerola-derived vesicle-nucleic acid drug complexes and their efficacy as nucleic acid drugs. PKH26-labeled acerola vesicles were orally administered to mice, and the pharmacokinetics were observed at 1, 3, and 6 hours after administration. Fluorescent signals were observed in the stomach, intestinal tract, liver, and kidney at 1 hour after administration. Interestingly, signals were also observed in brain tissue including the cerebrum, suggesting that the acerola vesicles also reached the central nervous system after oral administration.In addition, since orally administered nucleic acid drugs are likely to be absorbed in the portal vein, the liver is the primary target for investigation aimed at clinical application. Therefore, a complex of siRNA targeting the ApoB gene in the liver and acerola-derived vesicles was orally administered, and the knockdown efficiency of the ApoB gene in the liver tissue was analyzed by qPCR over time. The results showed that ApoB gene expression was decreased in the liver and small intestine 24 hours after oral administration, i.e., the knockdown effect by siRNA was confirmed. [Conclusions] The major features of this research are: 1) the focus on plant-derived exosomes, which are inexpensive and can be supplied stably; 2) the development of a highly safe drug (DDS formulation) by using plant-derived vesicles, which are usually edible by humans; and 3) the development of the world's first orally administrable nucleic acid medicine.

558 VecTabs Target Oxidized Phosphatidylcholine for Treating Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive loss of motor neurons, eventually leading to paralysis and premature death. It is a multifactorial disease characterized by protein aggregation, neuroinflammation, mitochondrial dysfunction, axonal defects and neuromuscular junction (NMJ) damage. Most ALS patients are diagnosed with sporadic ALS (sALS), with no apparent genetic contribution identified. Nonetheless, native TDP-43 aggregation is found in 97% of all patients. Besides TDP-43 pathology, one of the strongest metabolic abnormalities in affected ALS motor neurons is the dramatic increase of glycerophospholipids. Certain glycerophospholipids, such as phosphatidylcholines (PC), are enriched in ALS biofluids, correlated with disease progression and can be oxidized hence forming neurotoxic oxidized phosphatidylcholines (OxPCs). In fact, OxPC deposits are found in the axon segment of ALS motor neurons. Accumulating evidence has shown that cerebrospinal fluid (CSF) from sALS induces a drastic ALS phenotype in-vivo, and that this toxicity is low density lipoprotein (LDL)-mediated. Since LDL are not toxic themselves and has been associated with ALS progression, we hypothesize that OxPCs contained in OxLDL are the propagating vehicle of degeneration in sALS. Motor neuron exposure to OxPC triggered ALS-like phenotypes at transcriptomic level. Here, we describe a therapeutic strategy to target neurotoxic OxPCs in the context of ALS. We have generated a VecTab which is able to bind OxPCs and neutralize their pathological effects. OxPC VecTab prevented OxPC toxicity in iPSC motor neurons. Furthermore, biodistribution studies in large animals demonstrate robust transduction and expression of VecTabs in cortical, spinal cord, and brain stem regions following intracisternal administration. Finally, we show OxPC VecTab can potentially protect neuronal cells against OxPC toxicity.

559 Effects of Urine Stem Cell Derived Extracellular Vesicles on a Human Kidney Organoid Model of Acute Kidney Injury

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Acute kidney injury is associated with morbidity and mortality, necessitating the development of new therapies to improve patient outcomes. Technological and clinical advancements in AKI treatment have supported patient recovery, however most treatment options are supportive or supplementary and not curative. USCs are adult stem cells of renal origin that can easily be obtained through noninvasive methods. USCs are highly proliferative compared to other stem cell types. EVs from stem cells have been shown to elicit regenerative functions, while those secreted by diseased cells can serve diagnostic purposes. EVs elicit their effects with high specificity due to integral membrane proteins that interact with target cells upon contact. We thus, hypothesized that USC-EVs have the greatest potential for treating AKI because they are secreted from kidney stem cells. Here, we use a cisplatin-induced nephrotoxicity model to mimic AKI. Cisplatin is a chemotherapeutic agent used to treat several cancers including testicular, bladder, ovarian, esophageal, breast, and other cancers. Developments in kidney organoids derived from human induced pluripotent stem cells (hiPSCs) have provided an improved in vitro model for kidney diseases in recent years. Cisplatin-induced AKI kidney organoid models show tubular toxicity, suggesting their

relevance as a disease model. Our data demonstrated that in vitro treatment with USC-EVs reduced tubular injury and oxidative stress from nephrotoxicity. Additionally, our data show that USC-EVs increase staining for Ki67, a biomarker for proliferation. The results from our study suggest potential of USC-EVs in promoting cellular regeneration through proliferation.

560 ARMMs as a Versatile Non-Viral Delivery Platform for Therapeutic RNA Molecules

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One of the major challenges of RNA-based therapeutics is the efficient packaging and functional delivery of RNA molecules. Major limitations of existing delivery platforms include achieving efficient packaging and transfer of functionally active RNA cargos in recipient cells, without eliciting unwanted immune responses. Overcoming these barriers would expand the potential of RNA therapies. We engineered ARMMs (arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles), a class of extracellular vesicles that bud directly from the cell surface, to package a variety of labile RNA molecules, including shRNA, mRNA, and aptamers. Here we present data demonstrating functional delivery of various types of RNA via engineered ARMMs. We developed a system that permits active non-covalent recruitment of RNA payloads using ARRDC1, or engineered variants thereof, as an active loading handle into ARMMs. In this body of work, we demonstrate functional delivery and activity of RNA molecules by ARMMs in recipient cells. ARMMs loaded with shRNA molecules induce robust reduction of target mRNA and corresponding protein upon delivery in multiple cell types. P53 mRNA delivered via ARMMs induces activation of p53 target genes in p53-null recipient cells. Further, our data show that uptake of ARMMs loaded with RNAs is dose-dependent, without toxicity, and in contrast to other delivery systems does not trigger innate immune pathways, including those mediated by TLR7, in recipient cells. Our data support development of therapeutic strategies enabled by ARMMs-mediated intracellular delivery of RNA molecules.

561 Scalable Suspension Cell-Based Production and Purification of Engineered ARMMs as a Platform for Non-Viral Therapeutics

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A variety of human extracellular vesicles are currently being evaluated as promising platforms for non-viral delivery of therapeutic payloads. Scaling production, purification, and characterization of extracellular vesicles remains a major limitation. We are engineering a class of extracellular vesicles called ARMMs (ARRDC1-mediated microvesicles) as vehicles for delivery of protein, RNA, or ribonucleoprotein complexes. Here, we report a scalable approach for suspension cell-based production of ARMMs paired with an optimized downstream purification process. Using our cell-based production platform, we loaded ARMMs with various payloads including Cre recombinase, Cas9/guide RNA complexes, or short hairpin RNA. We evaluated production in 1 and 5 L shake flasks and in a fed batch stirred tank bioreactor. Three days following transfection of ARMMs-loading constructs, conditioned media were harvested and subjected to downstream purification processes. We evaluated multiple approaches to purify ARMMs by tangential flow filtration and mixed-mode size exclusion chromatography or monolithic anion exchange chromatography. We further subjected ARMMs loaded with Cre protein to stability testing before and after purification, followed by evaluation of potency. Our data establish robust and scalable production and purification processes for ARMMs with multiple payloads. These advances support the potential to utilize these processes for manufacturing of ARMMs to support clinical trials.

562 Novel Approach for Fluorescent Labeling of Intraluminal Protein Payloads in ARMMs as a Model Extracellular Vesicle

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Modern extracelullar vesicle (EV) characterization often utilizes fluorescent labelling of proteins, lipids, nucleic acids, or structural components. Labelling of internal EV proteins often requires transgenic co-expression of fluorescent proteins. Here, we present two protocols which allow targeted fluorescent labelling of lumen-side EV proteins, using ARMMs (ARRDC1-mediated microvesicles) as a model EV, and demonstrate detection efficiency as "percentage of targets labelled" through the use of nano-flow cytometry (nFCM). HEK (Human embryonic kidney) 293 suspension cells were transfected with a variety of ARMMs loading constructs and EVs were enriched from supernatant of transfected cells using size exclusion chromatography (SEC). Presence of ARMMs was confirmed by Western blotting and quantified by nFCM using a NanoAnalyzer (optical technique suitable for objects 40-1000nm diameter). Transgenic protein loading was assessed by nFCM as was subsequent fluorescent labelling. Intraluminal localization of the transgenic proteins was confirmed by anti-GFP, anti-SPOT tag, anti-FLAG tag fluorescent labelling. ARMMs loading of GFP tagged with SPOT from transgenic lines was shown to be ~70% within EVs >40nm. Fixation and/or permeabilization was shown to have limited impact on particle size and concentration, with EVs retaining a median size between 60-70nm. Incubation with anti-SPOT nanobodies following permeabilization demonstrated that up to 62% of the GFP labelled EVs could be further labelled in two unique protocols. Comparison between antibody and nanobody labelling of internal proteins demonstrated the superior detection efficiency of using smaller targeting reagents, such as nanobodies. Length of incubation, fixative concentration and starting EV concentration were all shown to impact efficiency of detection of EV payloads. Here, we describe a novel approach to test the efficiency of immunodetection of intraluminal payloads in EVs and demonstrate consistency with

payloading using a quantifiable internal fluorescent protein. We provide two viable protocols for internal labelling with distinct advantages and a potential model to continue exploration of novel approaches.

563 Rational Design of Multivalent Ionizable Lipid Delivery Systems for mRNA Delivery to Blood Cells

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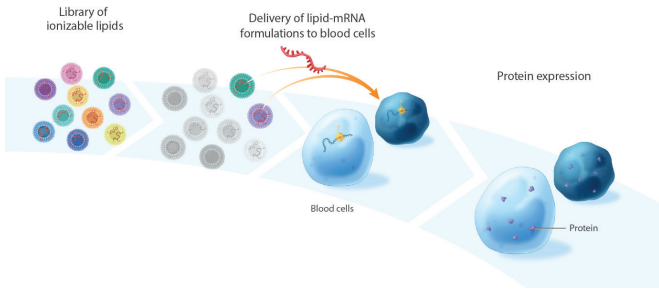
In recent years, lipid-based mRNA delivery has become the gold standard method for inducing exogenous protein production *in vivo* as evidenced by the success of the COVID-19 mRNA vaccines. While intramuscular injections are ideal for vaccine applications, intravenous injections are generally more suitable for achieving broad internal distribution of therapeutic payloads. Following systemic administration, blood cells are the first cells encountered by lipid nanoparticles (LNPs) and therefore serve as high interest targets for *in situ* protein production. With the goal of identifying a lipid formulation capable of efficiently transfecting blood cells, we synthesized and screened a library of 16 multivalent ionizable lipids with variations in headgroup and lipid tail. Headgroup variations included spermine (a naturally occurring biomolecule), dihydroxyspermine, 2-hydroxypropylamine, and ethanolamine (the headgroup of SM-102, the ionizable lipid component of Spikevax). The lipid tails varied in degree of unsaturation, carbon chain length, and head-to-tail spacer length. Lipids were characterized as lipoplexes and as solid LNPs, using mRNA encoding green fluorescent protein (GFP) as representative nucleic acid cargo. Nanoparticle size, surface charge, mRNA encapsulation, transfection efficiency, and cellular toxicity were evaluated. Lipoplexes were formulated at various weight ratios of lipid/mRNA, and mRNA-loading efficiency was determined via gel electrophoresis. The lowest lipid/mRNA weight ratio that displayed complete complexation for each lipid was used for the corresponding lipids in subsequent *in vitro* analyses. Notably, the greatest transfection efficiency in the lipoplex form was induced by FB3-54, a lipid comprised of a spermine headgroup and bis hexyl 2-hexyldecanoate tails. FB3-54 lipoplexes enabled efficient transfection of GFP mRNA in the THP-1 human monocyte cell line and exhibited a half-maximal effective concentration (EC_{50}) of 147 ng RNA per 50,000 cells, which was lower than that of Lipofectamine 3000 (242 ng RNA per 50,000 cells). The measured diameter of the FB3-54 lipoplexes was 359 nm. FB3-54 also functioned effectively as a solid LNP when using a molar ratio of 50:38.5:10:1.5 (ionizable lipid/cholesterol/DSPC/DMG-PEG_{2000}) and a 0.12 weight ratio of mRNA to ionizable lipid. FB3-54 LNPs were 170 nm in diameter, possessed a surface charge of +3.6 mV (at a pH of 7.0), demonstrated near complete encapsulation efficiency, and exhibited greater *in vitro* transfection efficiency than the clinically used Dlin-MC3-DMA LNPs. The ability to efficiently target blood cells using lipid delivery systems opens the door to a variety of applications, including potent *in vivo* mRNA delivery and cell reprogramming.



564 siRNA Treatment for Hereditary Tyrosinemia Type I (HT1)

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Hereditary Tyrosinemia Type I (HT1) is an autosomalrecessive disorder caused by mutations of fumarylacetoacetate hydrolase (*FAH*),the last enzyme of the tyrosine catabolism pathway. HT1 is inherited in anautosomal recessive manner affecting 1 in 100,000 newborns. Absence of FAHcauses the accumulation of toxic metabolites (such as succinylacetone, maleylacetoacetateand fumarylacetoacetate) that can damage the liver and the kidneys leading to acuteliver failure, renal disfunction and death, if untreated. HT1 management consistsof minimizing the built up of toxic metabolites by a very restrictive low tyrosine/lowphenylalanine diet in combination with nitisinone (NTBC), a small molecule drug that blocks4-hydroxyphenylpyruvate dioxygenase (HPD), an upstream enzyme in the catabolic pathway. Kidney damage and hepatocarcinoma (HCC) development are the most common long-termcomplications of this disorder when patients are not fully compliant, arecurrent problem in other metabolic disorders that require such a strictlifetime regimen. In recent years, liver-targetedsmall interfering RNA (siRNA)-baseddrugs have been approved for monogenetic disorders such as Hereditary ATTR(hATTR) Amyloidosis, Hypercholesterolemia, Hepatic Porphyria (AHP) and Primary HyperoxaluriaType I (PH1). HPD is a great target candidate for HT1 siRNA therapy since ithas been clinically demonstrated that its inhibition can prevent the diseasephenotype. We have previously shown that the deletion of the HPD genespecifically in the liver with CRISPR/Cas9 editing technology can rescue themetabolic phenotype of HT1 mice. In this study, we investigate theefficacy of HPD siRNA therapy in preventing FAH knockout mice from developingHT1 in the absence of nitisinone. All mice injected with HPD-siRNAs showlong-term survival, have low levels of succinylacetone and present no signs ofkidney toxicity.

565 Use of Low-Intensity Transcranial Focused Ultrasound for Targeted Delivery of Exosomes to the Brain

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The blood-brain barrier (BBB) presents a significant challenge for targeted drug delivery. In the present study, low-intensity focused ultrasound (LIfUS) was assessed for its ability to influence brain vessel endothelium, as well as its ability to increase the localization of small molecules when injected intravenously. Ultrasound is a well-established clinical technique for neurological diagnosis; however, LIfUS has recently been explored for its potential as a safe and controlled non-invasive, non-pharmacological technique for selective delivery of molecules up to 150 kDA to the brain that normally cannot bypass the BBB. A wide range of potential treatments are made possible by LIfUS, including directing exosomes to specified brain regions. When compared to stem cells, exosomes have high anti-inflammatory and regenerative properties, and low immunogenicity. Specifically, stem cell-derived exosomes have been shown to have multiple anti-inflammatory and neuro-restorative roles across multiple CNS indications including stroke, traumatic brain injury, Alzheimer's disease, and Parkinson's disease. This novel combination of applying pre-treatment with targeted LIfUS to directly localize intravenously delivered exosomes to specific region of the brain has high impact therapeutic potential to be deployed in a cost-effective manner in a clinical setting. In this study, LIfUS was applied in a targeted fashion to the right side of the rat brain to enhance the localization of intravenously administered labeled exosomes to the hippocampus. Our first set of results looking at the effect of LIfUS insonation on BBB integrity showed increased adhesion and transcytosis spread of dextran tracer in the endothelium. Further study results demonstrated a significantly increased concentration of exosomes in the right hippocampus when compared to the untreated contralateral (left) hippocampus, and when compared to the control group (brain untreated with LIfUS). Additionally, this study showed no evidence of pathological changes, edema, or hemorrhage. Further studies with larger sample sizes are warranted to support these results, with the goal of translation to patient trials.

566 Porous Silicon Nanoparticles Deliver Cas9 Ribonucleoprotein *In Vitro* and *In Vivo*

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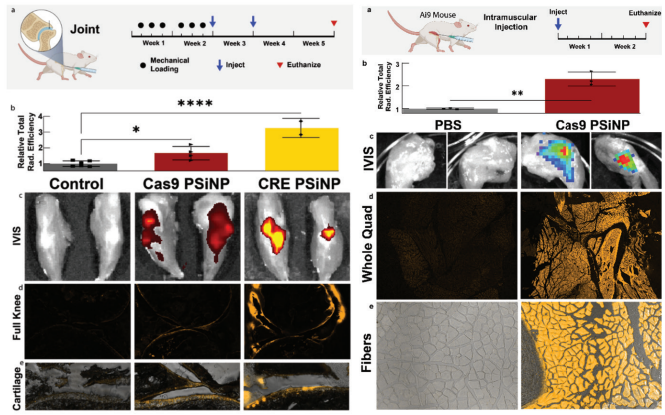
Background: Ex-vivo cell engineering has positively impacted medicine, yet has costly practical drawbacks well known to the field. Engineering cells in situ thus has serious clinical promise but remains an unsolved challenge due to limitations of viral manufacturing, safety, and non-viral material science. Porous Silicon Nanoparticles (PSiNPs) are a unique nanoscale material with interesting characteristics making them interesting as a gene delivery vehicle—specifically, they have been shown to load cargoes as diverse as DNA, RNA, PNA, proteins, and small-molecule drugs. PSiNPs are also fully biodegradable and well-tolerated. Prior work in our group has demonstrated that coating PNA-loaded PSiNPs in a polymer, PEG-DB, serves to stabilize the particles and facilitate endosomal escape into the cytosol. **Approach:** In this work, we optimized PegDB-PSiNP nanoparticles in Vitro for delivery of gene-editing proteins and demonstrated their In Vivo effectiveness after intraarticular, intramuscular, or intravenous administration. Additionally, we have begun optimizing for delivery of DNA for plasmid or donor-DNA applications. **Methods:** PSiNPs were verified by TGA and DLS. Loading of proteins was screening in vitro and verified by BCA assay and SDS-PAGE densitometry. In Vitro uptake and endosomal escape were verified by timecourse confocal microscopy. Gene-editing in vitro was screened using mTmG fluorescent reporter cassette in NIH-3T3 mouse fibroblasts and compared to Lipofectamine CRISPRMAX. In Vivo experiments were performed in Ai9 reporter (tdTomato inducible) mice and editing was verified by IVIS and cryohistology. **Results:** Optimizing the formulation in vitro resulted in endosomal escape ~10x, and DNA editing levels ~2x, that of Lipofectamine CRISPRMAX. Intraarticular administration resulted in DNA-editing in both the cartilage and synovium. Intramuscular administration resulted in nearly 100% positive muscle fibers at the administration site. Systemically administered Cas9-PSiNPs target to and edit in injured muscle at least as well as a commercial standard for mRNA—Polyplus in vivo jetRNA.



567 Intravenously Administered Carrier-Free Engineered Cas9 Ribonucleoproteins Target-To and Edit Inflamed Muscle

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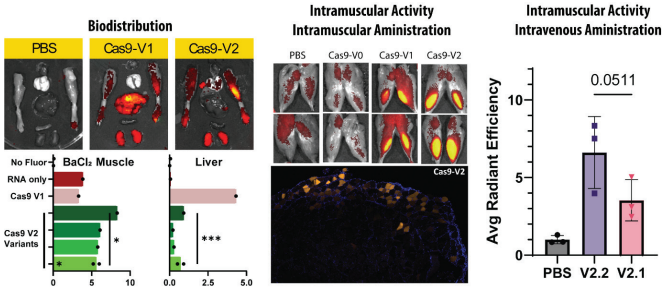
Background: Gene editing proteins are commonly regarded as incompatible with directly delivery, needing to be encapsulated and/or delivered in encoded (e.g., mRNA or AAV) form. However, this creates carrier-associated risks—be it AAV immunogenicity or cationic lipid toxicity. These issues motivate our pursue of a strategy

for "carrier-free" delivery. Carrier-free ribonucleoprotein (RNP) delivery for gene editing has been demonstrated for applications where the cargo is direct-injected intratumorally or intracranially. Systemic diseases, like Duchenne's Muscular Dystrophy (DMD), are not amenable to treatment with local injections. We seek to develop a carrier free RNP for systemic delivery to edit skeletal muscle, toward a therapy for DMD, a genetic disease characterized by muscle injury, inflammation, and degeneration. **Approach:** We hypothesized that, by targeting Cas9 RNP to sites of inflammation and avoiding liver accumulation, we could achieve gene-editing in inflamed muscle after intravenous administration. **Methods:** Engineered Chimeric Cas9 proteins were predicted using Alphafold, and then expressed in E. Coli and purified. Enzymatic activity was verified in vitro to explore structure-function relationships and narrow candidates for in vivo exploration. To explore biodistribution, guide RNA and/or Cas9 protein was fluorescently labeled and administered intravenously. To explore editing, Cas9 RNPs were formed to target Ai9 reporter (tdTomato inducible) mice. To explore local editing, Cas9 RNPs were administered intramuscularly to healthy muscle of Ai9 mice. To explore systemic editing, muscle inflammation was induced 2 days before RNP treatment by intramuscular administration of Barium Chloride. On day zero, Cas9 RNPs were administered systemically via tail vein. Ai9 mice were sacrificed after 1 week and analyzed via IVIS imaging. **Results**: Engineered Cas9 Variants were enzymatically active. Preliminary IVIS results suggest that after intravenous injection, engineered Cas9 Variants (Cas9-V2) experienced an ~80% decrease in liver accumulation and an ~100% increase in injured muscle accumulation relative Cas9 engineered only to enhance delivery (Cas9-V1). Directly injected into muscle, a Cas9 engineered only to enhance delivery (Cas9-V1) had >6-times the editing signal of a un-engineered Cas9 (Cas9-V0). Cas9 engineered to both enhance delivery and targeting (Cas9-V2) had >20 times the editing of standard Cas9. After intravenous administration, an engineered Cas9 variant had significantly increased IVIS editing signal over baseline. Further work is ongoing thanks to the sponsorship of Sarepta Therapeutics, Inc.



568 Efficient Delivery of Nucleic Acids by Lipid Nanoparticles to the Liver, Spleen, and Brain

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The delivery of genetic drugs such as mRNA or plasmid DNA has remained elusive yet critical for the development of gene-editing therapeutics. Viral delivery methods that have been traditionally used present the risk of uncontrolled expression of the delivered genes. Thus, non-viral delivery methods that can enable transient gene expression are desired. mRNA delivery via lipid nanoparticles (LNPs) has been successfully used in clinics marking a milestone for mRNA therapeutics. In this study, we generated and tested four different LNP formulations composed of the multivalent cationic and neutral lipid headgroups. We screened these formulations by delivering various mRNA in vitro and in vivo. For in vitro studies, we tested the efficacy of these formulations by delivering chemically modified sgRNA and Cas9 mRNA on neuronal primary cells and Cre mRNA on brain organoids. Furthermore, we successfully delivered luciferase mRNA in vivo by intravenous, intramuscular, intraperitoneal, and subcutaneous injections. Across in vitro and in vivo studies, our formulation showed better transfection efficiency compared to the standard FDA-approved D-Lin formulation. Of the four formulations, the multivalent cationic LNP demonstrated efficient mRNA and plasmid DNA co-delivery in vitro. Our results show these formulations can efficiently deliver nucleic acids to various organs, offering a promising platform for treating or curing muscle, skin, liver, spleen, and brain diseases.

569 Exploring Avenues Beyond the Liver: Optimization of Fusogenix Proteolipid Vehicles for Safe and Effective Delivery of Nucleic Acid

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Despite the promise of genetic medicines to treat various diseases, their success has been limited by challenges in delivering nucleic acids effectively and tolerably. Lipid nanoparticle (LNP) delivery platforms have undergone incremental improvements leading to commercial success, but still face issues with cellular toxicity and a biodistribution profile which is limited to the liver. To address these concerns, we developed a proteolipid vehicle (PLV) made from a chimeric fusion-associated small transmembrane (FAST) protein and a well-tolerated lipid formulation. We investigated whether extra-hepatic delivery could be achieved without the need for external targeting ligands by examining the effect of modifying the biophysical properties of FAST-PLVs on biodistribution. PLVs were manufactured using different parameters such as helper lipid composition and ratios, N/P charge ratio, PEGylation, and microfluidic flow rate, and we were able to achieve dramatic changes in size and physicochemical properties, irrespective of the encapsulated cargo (DNA, mRNA, siRNA). Modulating these parameters altered the affinity of FAST-PLVs to a variety of extra-hepatic tissues, such as muscle, lungs, and kidneys, and improved the efficacy of gene expression (DNA/mRNA) or knockdown (siRNA) in target tissues. We then set out to compare the performance of FAST-PLVs to three clinical LNP formulations (MC3-LNPs, Pfizer-LNPs, and Moderna-LNPs) via different routes of administration (ROA), such as intramuscular, intrathecal lumbar, intracerebroventricular, and intravitreal injections. The injection of FAST-PLVs through these routes of administration (ROA) resulted

in robust localized expression of FLuc in the expected organ. For example, intrathecally administered FAST-PLVs were effectively distributed throughout the central nervous system and achieved high FLuc expression in multiple brain regions. However, all three conventional LNP formulations showed considerable liver-tropism, resulting in significant FLuc expression in the liver, regardless of the ROA. In contrast, FAST-PLVs did not show any off-target FLuc expression irrespective of the ROA. This was associated by a favorable safety profile, as mice administered with FAST-PLVs had significantly lower circulating cytokine levels and showed no signs of pathology in histological analysis. Taken together, we show that extra-hepatic delivery of nucleic acids can be achieved by FAST-PLVs, and that they lack off-target effect, making them a safe and promising platform for the clinical development of genetic medicines.

570 Development of Multi-Functional Anti-Tumor E. coli for Cancer Therapy

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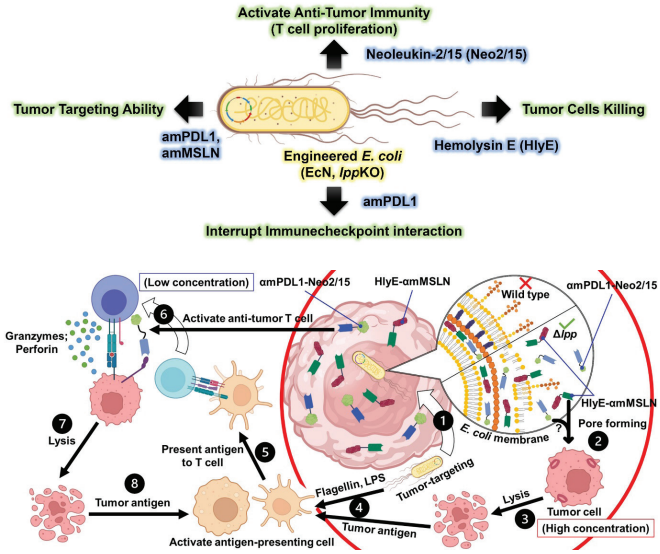
Non-pathogenic *Escherichia coli* (*E. coli*) is one of the microbes that has shown promising efficacy in cancer therapy. Benefiting from the immunosuppressive environment, *E. coli* can target and colonize primary and metastatic tumors in mouse models and compete with cancer cells for resources. However, bacterial therapy using wild-type *E. coli* can only moderately inhibit tumor growth without complete eradication. We plan to design interleukin-2 (IL2) variants secreted from *E. coli* and evaluate their anti-tumor ability. The recombinant IL2, which stimulates the proliferation of lymphocytes, was the first FDA-approved cytokine for cancer therapy, including malignant melanoma and renal cell carcinoma. Unfortunately, IL2 also activates the regulatory T (Treg) cells and inhibits the tumoricidal function of cytotoxic T lymphocytes (CTLs). Neoleukin-2/15 (Neo2/15) and superkine-2 (Sk2) are artificial IL2 variants that show better CTL proliferation over Treg in mouse models. Our mouse experiments indicated that the intratumoral injection of Neo2/15-secreting *E. coli* significantly suppressed the tumor growth. Further characterizations revealed that the number of TILs significantly increased after treating with the Neo2/15-secreting *E. coli*. We also found that the cured mice had induced immunological memory, as the re-challenges of the cancer cells did not develop any tumors. To further strengthen the anti-tumor ability of *E. coli* especially through the intravenous administration, we developed a multifunctional anti-tumor *E. coli* (MATE). MATE integrates the functions of tumor targeting, tumor killing, and immune activation by expressing multiple therapeutic proteins, including Neo2/15, anti-PDL1, and hemolysin E (HlyE). We demonstrated *in vitro* that MATE can efficiently kill the cancer cells as well as stimulate the T-cell proliferation. More importantly, MATE also exerted excellent anti-tumor activity via the intravenous injection in the mouse model. This work provides an early successful example of integrating multiple anti-tumor strategies into one bacterial entity for cancer therapy.



571 Lipid Nanoparticle Composition Shapes Immune Response to mRNA Vaccine and Potency of Anticancer Immunity

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Introduction: Lipid nanoparticles (LNPs) have been successfully designed as immunostimulatory delivery platforms for antigen-encoding mRNA, as exemplified by the LNP-based COVID-19 vaccines. However, there is a lack of systematic investigation into the effect of LNP composition on properties and efficacy of the immune responses elicited by mRNA LNP vaccines. Here, we developed a multi-step screening method to optimize the type of helper lipid and component ratios in an LNP formulation to efficiently deliver antigen-encoding mRNA to antigen presenting cells for vaccine-based cancer immunotherapy. By screening *in vitro* transfection activity, dendritic cell (DC) maturation and antigen presentation, and *in vivo* for immune activation and suppression of tumor growth, LNP formulations with potent antitumor efficacy are investigated as delivery systems for anti-cancer mRNA vaccines.**Methods:** To generate a 1,080 formulation LNP library, we used DLin-MC3-DMA as the ionizable lipid, cholesterol, DMG-PEG2000, and one of six helper phospholipids (DOTAP, DDAB, DOPE, DSPC, 14PA, and 18PG) that were previously used in FDA-approved or experimental LNP formulations. By varying the following parameters: (1) combined molar percentage of DLin-MC3-DMA and helper lipid; (2) weight ratio of cholesterol to DMG-PEG2000; (3) weight ratio of DLin-MC3-DMA to helper lipid; and (4) the molar ratio of chargeable groups in ionizable lipid to phosphate groups in pDNA (N/P ratio), a series of LNP formulations were generated using mRNA to screen *in vitro* for transfection efficiency in DCs. Selected LNP formulations were then loaded with ovalbumin (OVA) mRNA to assess the ability of antigen presentation, and induction of co-stimulatory molecule expression by DCs. LNPs with the highest DC transfection, antigen presentation, and maturation were further tested *in vivo* for transport to the draining lymph nodes (dLNs) via subcutaneous (s.c.) injection. LNPs showing effective transfection in dLN cells were administered s.c. into C57BL/6J mice to evaluate the immune responses

and anticancer efficacy in prophylactic and therapeutic treatment models of melanoma.**Results:** Based on the initial library screening, top-performing LNP formulations exhibited higher *in vitro* transfection efficiency in DC2.4 cells were tested in freshly isolated bone marrow-derived DCs (BMDCs) for antigen presentation and maturation analyses (Fig. 1A). The antigen presentation and maturation levels of APCs in dLNs were examined after s.c. injection of selected LNPs (C10, D6, and F5) (Fig. 1B). These three LNPs showed higher SIINFEKL-H-2Kb+ DC levels in dLNs and were tested for their vaccination efficiency. A significantly higher numbers of Th1 cells (CD4+IFN-γ+) were observed for all three formulations (Fig. 1C), though C10-treated group also showed the highest Th2 response along with a high OVA-specific IgG titer (Figure 1D-E). All three formulations are then tested on B16-OVA model and showed strong tumor growth inhibition efficacy with a prolonged overall survival time (Figure 1F). Furthermore, compared to D6 and F5, which generated strong Th1 response only, C10 LNPs, triggering both Th1 and Th2 responses, yielded a markedly improved protection effect.**Conclusion:**This LNP screening platform allows for identification of the best-performing mRNA LNPs for APC-specific gene expression, and tuning Th1/Th2 skewed immune response. Among the top candidates, C10 elicits potent Th1 and Th2 responses and mediates most potent antitumor efficacy compared to formulations with only Th1-skewed response.

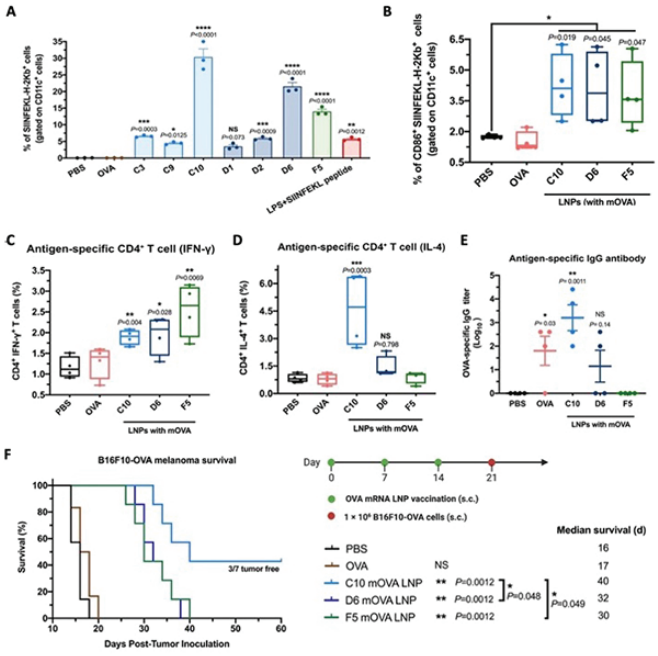

Figure 1. (A-B) SIINFEKL-MHC-I presentation by DCs treated with OVA mRNA LNPs *in vitro* (A) or *in vivo* (B). (C-E) Intracellular staining for IFN-γ (C) and IL-4 (D) in CD4+ T cells, and OVA antibody (E) after vaccinated with OVA mRNA LNPs. (F) Mouse survival following a prophylactic vaccination model for OVA-B16-F10 melanoma in C57BL/6 mice (n = 7; 10 μg mOVA per injection).

572 A PSMA Neovasculature-Inducible CA9 CAR Resistant to FASL and TGFB Mediated Suppression for the Treatment of ccRCC

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Clinically effective CAR-T cell therapy for solid tumors , will require substantial T cell engineering to increase their specificity and potency. We have developed an Integrated Circuit T cell (ICT) that encodes multiple synthetic "modules" in order to overcome diverse barriers to efficacy in clear cell renal cell carcinoma (ccRCC) ICT cells are generated via CRISPR-mediated, targeted knock-in of a single large transgene into a newly identified safe-harbor locus (GS94). Both primary and metastatic sites of ccRCC are highly vascularized, with the majority of tumor cells expressing elevated levels of carbonic anhydrase IX (CA9), suggesting CA9 may be an excellent CAR target. However, CA9 is also expressed in healthy bile ducts and stomach tissue which has led to on-target, off-tumor toxicities in patients treated with constitutive CA9 CAR T cells. To improve the therapeutic index of CA9 CAR T cells, we developed an "AND" logic gated ICT cell that requires the presence of two antigens to trigger tumor cell killing, thereby enhancing tumor specificity. Induction of the CA9 CAR is gated on the expression of prostate specific membrane antigen (PSMA) found on the tumor neovasculature of ccRCC. Importantly, PSMA and CA9 are not co-expressed in normal tissues. When the anti-PSMA priming receptor (PrimeRTM) binds PSMA, PrimeR engagement triggers proteolytic release of a chimeric, fully human transcription factor that induces expression of a CA9 CAR. We confirmed the feasibility of vascular priming using a transwell assay where ICTs were primed by a PSMA-expressing endothelial cell line and then migrated across the transwell membrane to kill CA9 expressing RCC cells. In addition, a dual flank xenograft model was used to show logic gated circuits selectively kill tumors that express both CA9 and PSMA, and not tumors that express CA9 alone. Transforming growth factor beta (TGFb) is an immunosuppressive cytokine known to be highly expressed in ccRCC. To further increase the potency and persistence of the ICT cells, an shRNA cassette was developed targeting both FAS and TGFBR2, a receptor required for TGFB signaling in T cells. Addition of FAS/TGFBR2 shRNA enhanced antitumor activity of PSMA x CA9 logic gate expressing T cells during in vitro chronic stimulation assays conducted in the presence of exogenous TGFb. Furthermore, FAS/TGFBR shRNA containing ICTs demonstrated enhanced anti-tumor activity in multiple xenograft RCC models. Collectively, these results demonstrate that PSMA x CA9 ICT cells can (i) selectively target antigens that cannot be safely targeted by conventional CARs and (ii) overcome multiple suppressive mechanisms in the tumor microenvironment.

573 An Oral Cancer Vaccine Using a *Bifidobactrerium* Vector Displaying a WT1 Antigen Protein for the Treatment of Urothelial Carcinoma

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Cancer immunotherapy with immune-checkpoint inhibitors (ICIs) including PD-1/PD-L1 inhibitors has been well established for various types of cancer including urothelial carcinoma (UC). However, monotherapy with ICIs can only achieve a durable response in a subset of patients. There is a great unmet need for ICI-resistant tumors and combining ICIs with cancer vaccines which forcibly induce an antitumor T cell response is a reasonable strategy. Previously, we generated a *Bifidobacterium* (*B.*) *longum* displaying a WT1 tumor associated antigen as an oral cancer vaccine. In our pre-clinical study, we explored the feasibility of this oral vaccine in a C3H/He mouse UC model (*Molecular Therapy: Oncolytics*, Vol. 22, 593-, 2021). A recombinant *B. longum* 420 displaying a partial murine-WT1 protein and an MBT-2 mouse UC cell line endogenously expressing WT1 protein were used in the study. Results reveal that a combination of oral administration of 1.0×10^9 colony-forming units (cfu) of *B. longum* 420, 5 times a week for 5 weeks, and intraperitoneal injections of 200μg of anti-PD-1 antibody twice a week for 2 weeks, completely suppressed the MBT-2 tumor growth and cured all mice tested, while all mice treated with anti-PD-1 antibody alone died. Furthermore, CD4 and CD8 positive T cells significantly increased in the tumor-infiltrating lymphocytes (TILs) of combination treatment group. Tumors which continuously grew despite the initial anti-PD-1 treatment were selected as anti-PD-1 antibody-resistant tumors. In the anti-PD-1 antibody-resistant tumor model, this vaccine alone significantly inhibited the tumor growth, while combination with continuous anti-PD-1 antibody did not demonstrate significant tumor growth inhibition. Interestingly, the number of regulatory T cells in TILs of the vaccine alone group was significantly lower than the combination treatment group. These results suggest that this oral cancer vaccine alone or as an adjunct to anti-PD-1 antibody could provide a novel treatment option for patients with advanced UC. From January 2023 at Kobe University Hospital, we started a Phase I clinical study (jRCT registered# 2051220143) of B440 (a lyophilized recombinant *B. longum* 440 displaying a partial human WT1 protein) monotherapy in patients with advanced UC resistant to PD-1/PD-L1 inhibitors. A total of 12 patients will be enrolled in this study and they will receive oral administration of B440 800mg or 1600mg, 5 times a week for 4 weeks. B440 is encapsulated in acid-resistant hard capsule, and one capsule contains 200mg of B440 equivalent to 2×10^{10} cfu of *B. longum* 440. In this Phase I study, safety (dose limiting toxicity) and efficacy (response rate, progression free survival) will be evaluated along with immunological parameters and bacterial compositions of the intestinal flora of participants. In this presentation, we will present a progress report of the Phase I study as well as the results of preclinical studies.

574 CAR-NK Cells Can Mediate Inflammatory Toxicities *In Vitro*

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Chimeric antigen receptor expressing NK (CAR-NK) cells are increasingly being developed to treat wide variety of human diseases. Unlike CAR-T cells, which are known to cause inflammatory toxicities such as cytokine release syndrome (CRS) and neurotoxicity, CAR-NK cells are thought to be safer based on limited published clinical data. For example, in a small clinical study (N=11), following administration of HLA-matched CD19 CAR-NK cells, none of the patients developed CRS or neurotoxicity. However, another study found CAR-NK cell therapy can cause inflammatory toxicities in some patients. Thus, it is unclear if CAR-NK cells are universally less inflammatory or its potential to cause inflammatory toxicities is dependent upon manufacturing factors such as NK cell source material, manufacturing process, or patient-specific factors such as disease type, tumor burden etc. Here we studied NK and T cells from same individuals (N=6) to assess if NK cells are less inflammatory than T cells, and to identify any factor(s) that may contribute towards inflammatory toxicities during CAR-NK cell therapy. We found that following activation, NK cells secrete significantly less inflammatory cytokines (IFN-γ and GM-CSF) compared to T cells from the same individual. Next, we compared NK and T cells for their ability to activate myeloid cells (MCs) *in vitro* as a measure to cause inflammatory toxicities. MCs treated with activated NK cell supernatant produced significantly less pro-inflammatory cytokines (IL-6 and IL-1β) compared to T cells. However, we did find that NK cells can activate MCs *in vitro*. Next, we sought to identify factors released by NK cells that activate MCs. Activated NK cell supernatant was subjected to size exclusion columns, and various fractions of different sizes were obtained. Each fraction was tested for MC activation. MC activation was significantly greater in the higher molecular weight (MW) fractions (>100kDa) compared to the lower MW fractions (<100kDa). Next, each of these fractions were subjected to the cytokine array to identify cytokines present within these fractions. Using data from multiple independent cytokine arrays, and published literature, we identified 13 candidate proteins that may contribute towards MC activation. From extensive antibody-based neutralization studies, we identified four candidate proteins as primary mediators of MC activation *in vitro* and neutralization of these four proteins resulted in over 50% reduction in MC activation. Furthermore, neutralization of these four proteins did not significantly impact NK cell cytotoxic functions. Current studies are underway to further validate these findings and rationally engineer CAR-NK cells by knocking out these inflammatory proteins using CRISPR/Cas with goals to improve safety and efficacy of CAR-NK cell therapy. Our data suggest that although NK cells are less inflammatory than T cells, NK cells can cause MC activation and can contribute towards inflammatory toxicities.

575 Human Plasma Cells Engineered to Produce Bi-Specific T Cell Engagers Show *In Vivo* Anti-Tumor Efficacy

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Bispecific T cell Engagers (BiTEs) have emerged as an important tool for the management and treatment of human hematological malignancies. However, BiTEs suffer from problems that hamper more general use including short serum half-life and on-target toxicity in non-tumor sites. Advances in genome-engineering have enabled the generation of human plasma cells that secrete large quantities of therapeutic proteins and that are capable of long-term in vivo engraftment in humanized mouse models. As a next step towards clinical translation in cancer therapy, here we describe the development and testing of a CRISPR Cas9 genome engineering strategy to generate ex vivo genome-engineered human plasma cells that express high levels of either an anti-CD19 (blinatumomab) or anti-CD33 (AMG 330) BiTE. We show that human plasma cells engineered to express BiTEs are capable of meditating T cell activation and direct T cell killing of target cancer cell lines, as well as primary human cells expressing the targets *in vitro*. Furthermore, BiTE-expressing plasma cells are capable of eliciting tumor eradication *in vivo* following local delivery to implanted tumor cells. Finally, immunocompromised mice engrafted with anti-CD19-BiTE secreting plasma cells were resistant to expansion of $CD19^+$ patient derived leukemia, a model that mimics the use of the anti-CD19 BiTE blinatumomab as a bridge to hematopoietic stem cell transplant or potential long-term therapy. These findings support further research into genome engineered human plasma cells for use as a local delivery system of BiTEs for the treatment of leukemias, lymphomas, and possibly other cancers.

576 Single-Cell RNA-Sequencing Uncovers a Neuron-Like Macrophage Subset Associated with Cancer Pain

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Pain is a common complication of advanced cancer with yet defined mechanism (Ji et al, Science 2016). Better understanding the immunodynamics of tumor microenvironment may identify effective therapeutic target for cancer pain (Tang et al, Advanced Science 2022). Here, we discovered a direct mechanism of tumor-associated macrophage (TAM) for promoting de novo neurogenesis via a subset showing neuronal phenotypes and pain receptors expression associated with cancer-driven nocifensive behaviors. This subset is rich in lung adenocarcinoma associated with poorer prognosis. By elucidating the transcriptome dynamics of TAM with single-cell RNA-sequencing analysis, we discovered a novel phenomenon "Macrophage to Neuron-like cell Transition" (MNT) as a direct mechanism for promoting tumoral neurogenesis, evidencing by macrophage depletion and fate-mapping study in syngeneic lung carcinoma mouse models. Encouragingly, we detected neuronal phenotypes and activities of the MNT cells in vitro. Adoptive transfer of the MNT cells into NOD/SCID mice markedly enhanced their cancer-associated nocifensive behaviors. Importantly, we identified macrophage-specific Tcf3 as a pivotal regulator for promoting MNT at genomic level, its disruption effectively blocked the tumor innervation and cancer-dependent nocifensive behaviors in vivo. Thus, MNT may represent a novel therapeutic target for cancer pain. AcknowledgementThis study was supported by the Research Grants Council of Hong Kong (RGC 14106518, 14111019, 14111720); RGC Postdoctoral Fellowship Scheme (PDFS2122-4S06); The Chinese University of Hong Kong's Faculty Innovation Award (4620528), Direct Grant for Research (4054510 and 4054668), and Postdoctoral Fellowship Scheme 2021-22 (NL/LT/PDFS2022/0360/22lt).

577 Genome-Wide CRISPR Screen Identifies Factors Influencing Natural Killer (NK) Cell Activity Against NK-Resistant Tumor Models

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Natural killer (NK) cells are innate lymphocytes that demonstrate cytotoxic activity towards stressed-self and/or modified-self cells (i.e. cancer cells). NK cells demonstrate strong anti-leukemic activity and bone marrow infiltration by NK cells is associated with a good prognosis in Acute Lymphocytic Leukemia (ALL) patients. However, NK cell activity is decreased in some patients because of the development of resistance mechanisms and NK cell exhaustion. To develop new therapies or improve existing ones, we need to fully understand how NK cells respond and kill ALL cells and what are the genes and pathways involved in this response. In order to identify key genes regulating sensitivity or resistance to NK cell cytotoxicity, we performed a whole-genome CRISPR screen in 697 B-ALL cell line. Using the Yusa CRISPR library, we induced mutations resulting in the knock out (KO) of 18,010 genes in 697 cells. Then, we co-cultured those cells with primary activated NK cells, derived from the NK-cell Activation and Expansion System (NKAES), until we reached more than 80% of target cell death. In previous cytotoxic assays, we showed that 697 cells are resistant to NK cell lysis after 4h of co-culture but become more sensitive after 24h of co-culture. Considering these results and to reach 80% of cytotoxicity, we did two rounds of 24h of co-culture at a ratio 4:1 (effector : target). First, the MAGeCK Robust Rank Analysis in surviving cells identified the death receptor pathway as the major mechanism involved in the NK cell mediated lysis of 697 cells. In fact, sgRNA targeting *FADD*, *CASP8*, *FAS* and *TNFRSF10A* were enriched in resistant tumor cells. We generated the 697 cell line KO for *FADD* and confirmed with a cytotoxicity assay that loss of FADD decreased NK cell-mediated killing. Furthermore, as expected

the analysis of the depleted sgRNA in surviving 697 cells showed that loss of *HLA-E, HLA-C* and *B2M* increase their sensitivity to NK cells. HLA molecules are known ligands of NK cell inhibitory receptors, HLA-E is a ligand of NKG2A and HLA-C is recognized by the killer cell immunoglobulin receptors (KIRs). In summary, the preliminary results of our study suggest that in resistant B-ALL, induction of the death receptor pathway or blocking NKG2A and/or KIR receptors might be a solution to enhance the efficacy of NK cell based immunotherapy.

578 Harnessing CRISPR/Cas9 Mutagenesis Screening for Rational Design of Next-Generation CAR-NKT Therapy Against Neuroblastoma

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Background and aims: Natural killer T cells (NKTs) possess innate antitumor properties that provide advantages over conventional T cells for use in cancer immunotherapy. We found that NKTs expressing a chimeric antigen receptor (CAR) specific for neuroblastoma antigen GD2 showed better tumor infiltration and ability to modify the tumor microenvironment than GD2-CAR T cells. Genes that regulate NKT cell persistence, functional fitness, and central memory differentiation remain largely unknown. To address this gap, we have developed a CRISPR/Cas9 screening system to identify genes that regulate the persistence and antitumor activity of NKTs and GD2-CAR NKTs for therapeutic targeting. **Methods:** We have developed the first CRISPR/Cas9 screening system for use in human NKTs and GD2-CAR NKTs to identify genes that regulate survival and proliferation of these cells during *in vitro* serial tumor challenge. As part of this system, we generated a library of guide (g)RNAs (five per gene) specific for a panel of 1,117 immune-related and 48 non-targeting gRNA controls. We have developed methods for graded lentiviral transduction of NKTs/CAR-NKTs followed by multiplexed functional testing and next generation sequencing of gRNA-transduced cells. We also established a system to validate candidate genes that includes *in vitro* and *in vivo* functional assays. **Results:** Our first screening round in human NKTs yielded nine candidate genes of interest, out of which we have found that PRDM-1 knockout improved NKT persistence during *in vitro* serial tumor challenge and increased expression of CD62L, a marker of central memory differentiation that has been shown to correlate with improved anti-tumor activity of GD2.CAR-NKT cells in an ongoing phase 1 clinical trial in neuroblastoma patients (NCT03294954). **Conclusions:** A CRISPR/Cas9-based screening system is feasible for use in human NKTs/CAR-NKTs to identify novel genes that regulate persistence and antitumor potential of NKTs in an unbiased manner. Results obtained from this study will ultimately help to enhance the antitumor efficacy of NKT cell-based cancer immunotherapy.

579 An *Ex Vivo* 3D Tumor Microenvironment-Mimicry Culture to Study TAM Modulation of Cancer Immunotherapy

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Tumor-associated macrophages (TAMs) accumulate in the solid tumor microenvironment (TME) and have been shown to promote tumor growth and dampen antitumor immune responses. TAM-mediated suppression of T-cell antitumor reactivity is considered to be a major obstacle for many immunotherapies, including immune checkpoint blockade and adoptive T/CAR-T-cell therapies. An ex vivo culture system closely mimicking the TME can greatly facilitate the study of cancer immunotherapies. Here, we report the development of a 3D TME-mimicry culture that is comprised of the three major components of a human TME, including human tumor cells, TAMs, and tumor antigen-specific T cells. This TME-mimicry culture can readout the TAM-mediated suppression of T-cell antitumor reactivity, and therefore can be used to study TAM modulation of T-cell-based cancer immunotherapy. As a proof-of-principle, the studies of a PD-1/PD-L1 blockade therapy and a MAO-A blockade therapy were performed and validated.

580 Tumor-Localized Administration of α-GalCer to Recruit Invariant Natural Killer T Cells and Enhance Their Antitumor Activity Against Solid Tumors

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Invariant natural killer T (iNKT) cells have the capacity to mount potent anti-tumor reactivity and have therefore become a focus in the development of cell-based immunotherapy. iNKT cells attack tumor cells using multiple mechanisms with a high efficacy; however, their clinical application has been limited because of their low numbers in cancer patients and difficulties in infiltrating solid tumors. In this study, we aimed to overcome these critical limitations by using α-GalCer, a synthetic glycolipid ligand specifically activating iNKT cells, to recruit iNKT to solid tumors. By adoptively transferring human iNKT cells into tumor-bearing humanized NSG mice and administering a single dose of tumor-localized α-GalCer, we demonstrated the rapid recruitment of human iNKT cells into solid tumors in as little as one day and a significantly enhanced tumor killing ability. Using firefly luciferase-labeled iNKT cells, we monitored the tissue biodistribution and pharmacokinetics/pharmacodynamics (PK/PD) of human iNKT cells in tumor-bearing NSG mice. Collectively, these preclinical studies demonstrate the promise of an αGC-driven iNKT cell-based immunotherapy to target solid tumors with higher efficacy and precision.

581 Targeting WNT Signaling for Improved Glioma Immunotherapy

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Impressive results in a subset of cancers have been obtained using checkpoint immunotherapies including anti-CTLA4, anti-PD-1, and anti-PD-L1 or CAR-T therapies . However, the heterogeneity of tumors, low mutational burden, single antigen targeting, and associated antigen escape often contribute to non-responsiveness and potential tumor recurrence despite these therapeutic efforts. An important common mechanism of resistance observed in immunologically "cold tumors", including gliomas, involves aberrant activation of the Wnt/β-catenin signaling pathway. Enhanced tumor-intrinsic Wnt/β-catenin signaling appears to be a common mechanism mediating cancer immune evasion and is associated with the presence of an immunosuppressive cell subset as well as the prevention of effective dendritic cell presentation and T-effector cell recruitment and function. Increased expression of β-catenin inversely correlates with the presence of $CD8^+$ T cells and dendritic cells in multiple tumor types including glioma. Furthermore, Wnt pathway activation is correlated with tumor stemness and poor treatment outcome. The hostile tumor microenvironment (TME) is associated with decreased tumor antigen presentation, and greatly reduced or even lost efficacy of various therapies, including adoptive T cell immunotherapy. Therefore, targeted downregulation of Wnt/β-catenin signaling--thereby enhancing the response to immunotherapy in patients with relapsed and refractory tumors--is an attractive therapeutic approach. In the current study, we determined the effects of the small molecule, highly specific Wnt/CBP/β-catenin antagonist ICG-001 on glioma tumor cells and the TME--including immune cell infiltration, blood vessel decompression and metabolic changes. Differentiation of glioma cells and loss of glioma stem cells should enhance the antitumor immune response. We demonstrated *in vitro* cytostatic effects and a switch from proliferation to differentiation after treatment with ICG-001 using multiple patient-derived (PDX) glioma cell lines and murine tumors (GL261, K-Luc). We further demonstrated in these glioma cell lines that ICG-001 downregulated the CBP/β-catenin target gene, *survivin/BIRC5*--a hallmark of selective inhibition of Wnt/CBP/β-catenin signaling. Utilizing NanoString and proteomic analysis, we investigated the effects of ICG-001 *in vitro* on the patient derived glioma cell lines PBT030 and PBT147, and metabolic pathways regulated by Wnt signaling. Furthermore, we found that in a syngeneic mouse model of glioma (K-luc), ICG-001 treatment enhances tumor infiltration by $CD3^+$ and $CD8^+$ cells with increased expression of the vascular endothelial marker CD31 (PECAM-1) on days 7, 14 and 21 post tumor engraftment. We conclude that reprogramming the glioma tumor microenvironment by means of differentiation of glioma stem cells, modulation of the stroma and immune cell activation and recruitment, utilizing the pleiotropic effects of biochemically specific Wnt/CBP/β-catenin antagonists, may enhance immunotherapy in glioma patients.

582 Refining Chimeric Antigen Receptors via Barcoded Protein Domain Combination Pooled Screening

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Chimeric antigen receptor (CAR)-T cells are a rapidly emerging form of cancer immunotherapy, but the process for developing new CAR constructs is time-consuming and inefficient. To address this challenge, we developed a cloning strategy in which individual CAR domains are serially assembled and each domain includes a unique DNA barcode, allowing simultaneous quantification of CAR frequencies in pooled populations by next-generation sequencing. Using this method, we generated 360 CAR constructs that target clinically validated tumor antigens CD19 and GD2. We transduced T cells with these libraries and exposed the cells to CD19+ Raji or GD2+ CHLA255 tumor cells, then screened for constructs mediating superior proliferation and expansion by quantifying changes in barcode frequencies. In the process of evaluating individual domain contributions, we unexpectedly found that the effects of the hinge domain varied significantly between CD19- and GD2-based CARs. When comparing costimulatory domains, we observed that 4-1BB occurred more frequently than CD28 in the top-performing CARs. Importantly, the screen identified clinically validated CD19- and GD2-CAR constructs with either CD28 or 4-1BB domains among the top constructs. We did not detect significant differences between second- and third-generation CAR architectures, but top-performing third-generation CARs contained 4-1BB in the proximal position. Further characterization revealed that the dilute retroviral supernatant used for screening enhanced the anti-tumor functionality of CAR-T cells retrovirally transduced with a GD2/4-1BB construct both *in vitro* and *in vivo* mouse tumor models. Therefore, using our novel combinatorial method to assess the functionality of CAR constructs, we demonstrated the importance of structural domains including the hinge alone and in relation to the single-chain variable fragment, co-stimulatory elements and their position within the CAR, and transgene copy number in CAR-T cells for optimal CAR activity. This screening approach is readily scalable and has the potential to greatly accelerate the development of CAR-based cancer immunotherapies.

583 Transactivator-Free, Doxycycline-Inducible CAR-T Cells (iTRUCKs) for Improved Antitumor Efficacy

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Solid tumors treatment with Chimeric Antigen Receptor (CAR)-redirected T cells remains a challenge due to inhibitory tumor microenvironment (TME), poor tumor infiltration and poor CAR-T cells persistence. Arming CAR-T cells with immunomodulatory molecules (4th generation CAR-T cells or TRUCKs) could overcome these limitations but, at the same time, it will increase CAR-T toxicities. It is therefore crucial to develop inducible systems that can control the activity of these TRUCKs. We have previously developed an all-in-one transactivator-free doxycycline (Dox)-inducible, lentiviral vector (LVs) (Lent-On-Plus or LOP). Using these platform, we have generated the *first-in-class,* 4th generation anti-CD19-CAR-T cells engineered to release IL-18 only in presence of Dox and in the absence of transactivators (iTRUCK-1918). Antitumor potency of iTRUCK-1918 can be controlled with low Dox concentrations, improving antitumor potency against lymphoma and a CD19+ pancreatic cancer model, both *in vitro* and *in vivo*. Dox addition to iTRUCK-1918, increased T stem cell memory/T central memory phenotypes, reduced exhaustion markers and polarize M2 (pro-tumor) to M1 (anti-tumor) macrophages in a co-culture system. These results showed that LOP LVs can generate iTRUCKs that can be controlled with Dox *in vitro* and *in vivo*, constituting a promising tool for improving CAR-T cell potency, while controlling side effects.

584 Combination Treatment of Temozolomide + PARP Inhibitor Sensitize Ovarian Cancer Cells for Gamma Delta T Cell Killing through NKG2DL Expression

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With a five-year survival rate of less than 50%, ovarian cancer (OVCA) is the deadliest cancer impacting women. The most common and devastating histological subtype is high-grade serous ovarian cancer (HGSOC), comprising ~70-80% of deaths. While first-line surgery and platinum chemotherapy are initially effective, most cases recur. Over the last decade, poly ADP-ribose polymerase inhibitors (PARPi) have revolutionized HGSOC therapy and improved patient outcomes; however, recurrence remains a significant obstacle, with the emergence of cross-resistance to both platinum and PARPi. As such, new approaches are needed to tackle this unmet need. Alkylating agents such as temozolomide (TMZ) increase NKG2D-ligand (NKG2DL) expression on cancer cells through the DNA damage response (DDR) pathway, enhancing gamma-delta (γδ) T cell cytotoxicity. We hypothesized that the combination of PARPi + TMZ would synergize to further potentiate γδ T cell targeting in the context of HGSOC. We first determined the effect of TMZ monotherapy or in combination with the PARPi niraparib on the growth and viability of various OVCA cell lines. TMZ monotherapy did not show significant cytotoxicity in concentrations up to 300μM for the OVCA cells tested. OVCA cells are far more sensitive to niraparib monotherapy with LD30 ranging from 1.5 μM (OVCAR-8) to 9.63 μM (KURAMOCHI). In addition, the combined treatment of TMZ (100uM) and various concentration of niraparib showed enhanced cytotoxicity to the OVCA cells over that of single-agent treatment. We then examined 3 OVCA cell lines (OVCAR-3, OVSAHO, and KURAMOCHI) for DDR-induced upregulation of NKG2DL. NKG2DL expression was determined by flow cytometry for cells treated for 24h with vehicle, TMZ (200 μM), niraparib (5 μM) or the combination of TMZ and niraparib and expressed as % increase of median fluorescence intensity (MFI). Single-agent treatment with TMZ or niraparib upregulated NKG2DL expression (MIC-A, MIC-B, ULBP-1, and ULBP-2,5,6) between 8% and 60%. Interestingly, combination treatment of TMZ + niraparib resulted in a much greater increase (53% to 210%) of NKG2DL expression suggesting a synergistic effect. We cocultured the OVCA cell lines with activated and expanded Vδ2+ γδ T cells at increasing effector to target (E: T) ratios for 36 hours. γδ T cells demonstrated cytotoxicity against all three OVCA cell lines in a linear dose response manner. At E:T ratio 20:1, γδ T cells killed 63.4%±5.2%, 26.4%±6.0%, and 57.7%±3.8% of OVCAR-3, OVSAHO, and KURAMOCHI cells, respectively. These findings indicate that OVCA is a potential candidate extracranial tumor for concurrent infusion of chemo-protected DeltEx DRI γδ T cells + TMZ, currently in Phase I trials for glioblastoma (NCT04165941), along with adjuvant PARPi.

585 bbT369, a Clinical-Stage Dual-Targeted and CBLB Gene Edited Autologous CAR T Product for Non-Hodgkin Lymphoma, Shows Edit Driven Enhanced Activity in Preclinical *In Vitro* and *In Vivo* Models

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Anti-CD19 CAR T cell therapies have improved outcomes for non-Hodgkin lymphoma (NHL) patients. However, only about 30-40% of patients treated with commercially available CAR T cell therapies achieve long term remission, highlighting the need for more

efficacious and durable treatments. Emerging clinical data suggest several failure modes for CD19 CAR T cell therapies including: loss or downregulation of CD19 antigen, loss of co-stimulation pathways on tumor cells, exhaustion of CAR T cells, and immunosuppressive microenvironments. To overcome these hurdles, we devised the next-generation autologous CAR T cell therapy bbT369, currently in first-in-human Phase 1/2 trial (CRC-403 NCT05169489). bbT369 is a dual targeted (CD79a/CD20) CAR T cell therapy that uses an OR gate design to limit antigen escape, has split 41BB and CD28 co-stimulatory domain architecture to augment T cell activation, and contains a knock-out of the *CBLB* gene to enhance potency and reduce T cell exhaustion. Here we highlight aspects of our product design and performance, including demonstrating enhancement of CAR T activity derived from the CBLB edit in a variety of in vitro assays. We show that CD79a and CD20 expression is B cell lineage restricted in normal human tissue and confirm that these proteins are co-expressed in diffuse large B cell samples. To target these antigens, we developed a split dual-targeting CAR configuration and demonstrate it provides superior activity for bbT369-directed tumor cell killing of dual and single antigen positive tumor cells compared with traditional dual-CAR designs. To determine the importance of the CBLB edit on different aspects of CAR T cell function, we performed a suite of assays designed to model distinct challenges to maximal CAR T cell activity within the tumor, specifically: 1.) Chronic activation via a serial stimulation assay, 2.) Antigen downregulation via sub-optimal bead-based activation, 3.) Immunosuppression via T cell stimulation in the presence of recombinant TGFβ or plate-bound PD-L1, and 4.) Loss of costimulatory signaling via co-culture of T cells with CD58KO target cell lines. These data demonstrate that the CBLB gene edit directly contributes to enhancing the activity of the bbT369 CAR T cells across each of these *in vitro* model systems vs unedited controls. In addition, we show that bbT369 T cells drive greater T cell expansion and durable tumor control compared with unedited controls *in vivo* using a xenograft NSG mouse model. Collectively, the data support the potential for bbT369 to provide deeper and more durable responses in NHL patients including those with more challenging disease characteristics.

586 Effective Combination Immunotherapy Using onCARlytics and Artemis® CD19 T Cells Against Hepatocellular Carcinoma (HCC)

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Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and the sixth most common cancer worldwide. Currently, there are six systemic therapies available for patients with advanced disease including atezolizumab in combination with bevacizumab, lenvatinib, regorafenib, cabozantinib and ramucirumab while curative treatments include ablation, surgical resection, and liver transplantation. CD19 targeting chimeric antigen receptor (CAR) T cell therapy has demonstrated impressive clinical outcomes but translating this therapy to solid cancer has been met with various challenges, including the immunosuppressive microenvironment, on-target off-tumor toxicity, and antigen heterogeneity. To date, CAR T cell therapies against HCC targeting antigens such as alpha-fetoprotein and glypican-3 have shown nominal efficacy in clinical trials. Therefore, development of novel and innovative therapeutic approaches against HCC are desperately needed to overcome the challenges and improve clinical outcomes. Oncolytic viruses (OV) are a novel and attractive form of immunotherapy due to the ability to target tumor cells selectively, even in the absence of tumor specific antigens, and deliver genes of interest for therapeutic intervention. We have harnessed this capability of OV and developed a chimeric vaccinia-based OV called CF33-CD19t (onCARlytics) that delivers a non-signaling, truncated CD19 (CD19t) antigen to solid tumors allowing CD19-specific T cells to target them. In order to target CD19t expressed on the surface of solid tumor cells, we combined onCARlytics with CD19 ARTEMIS® T cells, a CD19-targeting adoptively engineered T cell powered by the ARTEMIS® antibody-T cell receptor (AbTCR) platform. The ARTEMIS® AbTCR distinguishes itself from traditional CAR by its recruitment of endogenous CD3 complex and utilizing the same activation and regulatory signaling pathways used by natural TCRs. Tumors infected with onCARlytics induced robust CD19t surface-antigen expression prior to virus-induced tumor lysis. Co-cultured CD19 ARTEMIS® T cells secreted cytokines and elicited potent cytolytic activity against infected HCC tumor cells. In addition, this combination approach demonstrated impressive *in vivo* anti-tumor responses in a human xenograft HepG2 tumor model. By using this combination OV and CAR T cell strategy, we have now broadened the utility of CD19 ARTEMIS® T cells to otherwise target-less tumors such as HCC, which we anticipate can be applied to a wide array of solid cancers as an effective immunotherapy approach.

587 Targeting TIM-3 for Metabolic Natural Killer Cell Activation Against Glioblastoma

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Background: Natural killer (NK) cells hold potential as one of the next generation adoptive cell therapy candidates due to their allogenecity, capability to lyse target without prior sensitization, and low risk of host-versus-graft disease. Thus, being able to utilize NK receptors to drive their cytotoxicity remains crucial. Additionally, for treatments of solid tumors, NK cells need to retain their viability against the suppressive microenvironment. One method is to leverage the surface receptor TIM-3, which lies at the intersection of NK cytotoxic functions and metabolism - the mTOR-associated pathways. **Methods:** Human NK cells were isolated from healthy adult peripheral blood, and expanded in K562-based feeder media. Flow cytometry was used to phenotype TIM-3 surface expression and phosphorylation of mTOR, Akt, and ribosomal protein S6 (rpS6). TIM-3 *knock-out* (TIM-3 KO) NK cells were generated by electroporating CRISPR/Cas9 ribonucleoprotein complexes, and subsequently cultured in 24-well G-Rex plates. Function and immune activation of TIM-3 *wild-type* and TIM-3 *KO* NK cell variants, in the presence or absence of mTOR inhibitors against patient-derived GBM43 were evaluated through target lysis, degranulation, and cytokine secretion. 3D tumor spheroids were

generated with GBM43 and 40 μg/mL collagen in 96-well ultra-low attachment plates, and NK cells were co-incubated for 4 days to test for long-term cytotoxicity. **Results:** We found that TIM-3 expression on *ex vivo*-expanded NK cells responded to inhibition of both mTOR complexes by torin-1 but not to single mTOR complex inhibition by either rapamycin or JR-AB2-011. Specifically, TIM-3 expression increased in response to torin-1, but did not change upon the addition of rapamycin or JR-AB2-011. To investigate this association, we challenged NK cells exposed to torin-1 against GBM43 *in vitro*, and observed that NK cells and torin-1 have co-operative toxicity against GBM43. We also noticed that TIM-3 *KO* NK cells did not exhibit similar co-operative effects, suggesting that the increase in target lysis could be attributed to the upregulation of TIM-3 expression. We also report changes in phosphorylation of different mTOR-associated proteins between TIM-3 *WT* and TIM-3 *KO* NK cells, which could contribute to changes in NK cell metabolism, ultimately leading to differences in cytotoxicity and cytokine production against 2D glioblastoma monolayer and 3D tumor spheroids. **Conclusions:** We determined that on *ex* vivo human NK cells, TIM-3 responds to mTOR activation as a possible means to protect NK cell metabolism, cytotoxicity and cytokine production in settings of metabolic inhibition. This protection of NK cells against mTOR inhibition, combined with our previously published data that TIM-3 upregulation increased production of FN-γ production, a cytokine that sensitize glioblastoma to NK-mediated lysis, afford an opportunity to increase the efficacy of NK cell therapy against glioblastoma by the addition of mTOR inhibitors without compromising any of NK cell functions.

588 Optimization of CAR T Cells Against the C Domain of Tenascin C for Pediatric Brain and Solid Tumor Immunotherapy

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Identifying safe, targetable antigens is a critical roadblock for the success of pediatric solid and brain tumor chimeric antigen receptor (CAR) T cells. To overcome this limitation, we developed a pipeline to identify exons upregulated in tumors when compared to normal tissues. A top candidate from our screen is the C exon from the long isoform of Tenascin C (C.TNC), an extracellular membrane (ECM) protein with high expression in pediatric osteosarcomas and gliomas. We verified C.TNC expression in diffuse intrinsic pontine glioma (DIPG), high-grade glioma (HGG), and pediatric sarcoma cell lines through RT-qPCR and IHC. Indeed, all evaluated cell lines express the splice variant, and in IHC images of primary tumors, there is a marked stromal deposition of C.TNC. We then generated a second-generation C.TNC-CAR with an scFv specific for C.TNC and a CD28z signaling domain. Using retroviral transduction of primary T cells, we achieve an average transduction efficiency of 66%. C.TNC-CAR T cells killed in an antigen and CAR-dependent manner in *in vitro* cytotoxicity assays. Upon antigen stimulation, C.TNC-CAR T cells produce significant amounts of cytokines and can eradicate tumor cells *in vitro*. With these data, we tested the C.TNC-CAR T cell effector function against established *in vivo* models of sarcomas and DIPGs. C.TNC-CAR T cells expanded transiently at tumor sites, resulting in limited antitumor activity. In an attempt to improve C.TNC-CAR T cell effector function, we first explored canonical changes to the linker, hinge, and transmembrane domains of the C.TNC-CAR. While several CAR constructs improved the effector function of CAR T cells *in vitro* as judged by IFNg production and cytolytic activity, this did not translate into improved antitumor activity *in vivo*. Therefore, we are currently investigating if providing cytokine support through the expression of leucine zipper-based constitutively active cytokine receptors (ZipRs) can improve the effector function of C.TNC-CAR T cells. *In vitro*, expression of ZipRs in C.TNC-CAR T cells enhances their ability to recursively kill tumor cells, and *in vivo* studies are underway. In summary, CAR T cells can target ECM proteins such as C.TNC and elicit an antitumor response. Our initial studies with C.TNC-CAR T cells warrant their further development for the immunotherapy of pediatric brain and solid tumors.

589 Monitoring FAP CAR T Cell Therapy with PET Imaging

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Two of the major obstacles that adoptive cell transfer immunotherapy needs to overcome to be successful in solid tumors are 1) the immunosuppressive tumor microenvironment (TME) and 2) the lack of robust biomarkers that allow for the identification of patients that would benefit from the therapy and for monitoring of the treatment response. Here we paired a CAR T cell therapy with a companion Positron Emission Tomography (PET) imaging approach that allows for serial, non-invasive, whole-body visualization of a biological target of interest -in this specific case, Fibroblast Activating Protein (FAP). FAP is a cell surface serine protease that is highly expressed by cancer associated fibroblasts in the TME that participate in the generation of the immunosuppressive stromagenic response in solid tumors. We designed a novel FAP CAR construct based on the scFv of the 4G5 antibody developed, in house, against canine FAP that cross-reacts against mouse and human FAP. In this study, we utilized the ^{18}F-radiolabeled FAP inhibitor (FAPI), [^{18}F]AlF-FAPI-74, to image FAP in two different mouse tumor models. First, the probe specificity was evaluated in the I45 human mesothelioma tumor model (a line which does not induce FAP^{+} fibroblasts). I45 WT and I45 cells transduced with human FAP were injected s.c. into the opposite flanks of a mouse and imaged following 2 weeks of tumor growth. We observed a 7.5-fold higher uptake of [^{18}F]AlF-FAPI-74 in the I45 huFAP tumor compared to the WT, demonstrating the high specificity of the probe for FAP. Next, we used a more clinically-relevant A549 model where the tumor cells do not express FAP but induce a stromagenic response and drive the

recruitment of FAP$^+$ stromal cells in the TME. [^{18}F]AlF-FAPI-74 PET/CT following 3 weeks of tumor growth showed a 6.5-fold increased radiotracer uptake in the tumor relative to the muscle at the baseline scan. Using this model, we evaluated the potential of our new CAR T cells to reduce tumor burden, as well as evaluating the potential of the [^{18}F]AlF-FAPI-74 tracer as a tool to monitor the clearance of FAP-expressing cells in response to FAP CAR T cell therapy. Immediately after the baseline scan, we injected 5x10^6 FAP CAR T cells iv. At day 14 post-T cell injection, mice treated with FAP CAR T cells had significantly smaller tumors relative to the control group, which were treated with T cells that do not express the CAR, highlighting the therapeutic efficacy of the FAP CAR T cell therapy. Moreover, [^{18}F]AlF-FAPI-74 PET/CT imaging showed no detectable tracer uptake in the tumors treated with FAP targeted CAR T. These findings were confirmed by immunofluorescence, indicating successful clearance of the FAP$^+$ stroma by the injected FAP CAR T cells. In conclusion, the new 4G5 FAP CAR shows specific targeting toward mouse stroma infiltrating lung adenocarcinoma xenografts. PET imaging is a powerful tool that can have may applications. PET imaging of FAP could be a highly useful approach to stratify patients prior to FAP CAR T therapy, as well as to monitor the pharmacodynamic response for FAP-targeted therapies.

590 CRTE7A2-01: A HPV-16 E7$_{11-19}$ Specific TCR Targeting HPV-Related Cancers

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Most cervical and anal cancers and over 25% of head and neck cancers is caused by infection with high-risk human papillomavirus (HPV). HPV16 is the most common high-risk HPV that induces cancers. HPV E7 oncoprotein is essential for HPV-induced carcinogenic transformation and is homogenously expressed in HPV-related cancer cells, these aspects make HPV E7 oncoprotein an ideal target for TCR-engineered T cell therapy. In a recent Phase I trial at the National Cancer Institute (NCI), an E7 TCR-T therapy (NCI-E7-TCR) showed a 50% objective response rate in heavily treated metastatic HPV-related cancers. By tetramer staining of expanded tumor infiltrating lymphocytes (TILs) form cervical cancer patients and CIN2 patients carrying the HLA-A*02:01 allele, we sorted T cells specifically recognized the HLA-A*02:01-restricted HPV16 E7$_{11-19}$ epitope with FASC single cell sorting. TCRα and β chains were obtained for each sorted single T cell by Corregene's Single T cell TCR Cloning platform. For each TCR candidate, the affinity, cytotoxicity, IFN-γ release, T cell proliferation and specificity were evaluated deeply by in vitro assays. We obtained a high-affinity TCR sequence that specifically recognized the HLA-A*02:01-restricted HPV16 E7$_{11-19}$ epitope: CRTE7A2-01. CRTE7A2-01 showed comparable affinity but better expression intensity and stability on T cells relative to the NCI TCR. Consequently, TCR-T engineered with CRTE7A2-01 outperformed T cells engineered with NCI-E7-TCR in all vitro assays. Moreover, CRTE7A2-01 displayed great specificity: no allo-reactivity evaluation against over 70 HLAs, no reactivity to a panel of over 30 HPV16-negative cell lines, and no off-target hits in extensive alanine scan(X-SCAN).In vivo experiments showed that CRTE7A2-01 TCR-T could significantly inhibit the growth of subcutaneous Hela tumors (overexpressing HLA-A*02:01 and HPV16 E7) in nude mice, greatly infiltrate into tumor tissues and persistently proliferated in peripheral blood. Furthermore, CRTE7A2 shows excellent safety in vivo. We are now conducting a single center, open, single arm dose escalation phase I study to evaluate the safety, tolerability, and efficacy of CRTE7A2-01 TCR-T cell for HPV16 positive advanced cervical, anal, or head and neck cancers. The study will determine MTD of CRTE7A2-01 TCR-T cell injection, as well as investigate RP2D (NCT05122221).

591 The Safety and Efficacy of Systemic Delivery of a New Liver-De-Targeted TGFβ Signaling Inhibiting Adenovirus in an Immunocompetent Triple Negative Mouse Mammary Tumor Model

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Aberrant TGFβ signaling has been shown to be negatively associated with disease-free survival (DFS) and overall survival (OS) of metastatic triple negative breast cancer (mTNBC), and positively linked to metastasis and tumor immune escape. Previously, we have found that oncolytic adenoviruses expressing a TGFβ signaling inhibitory protein (sTGFβRIIFc) inhibited bone metastasis in a human TNBC cell line (MDA-MB-231) immunodeficient intracardiac mouse model with systemic administration, and induced immune activation in a mouse TNBC cell line (4T1) immunocompetent subcutaneous model with intratumoral injection. Systemic administration of adenoviruses is predicted as the best route to treat mTNBC but faces the challenges of increased toxicity and viral clearance. To overcome these challenges, we have created a liver-de-targeted sTGFβRIIFc- and Lyp-1 peptide-expressing adenovirus (mHAdLyp.sT) with enhanced breast cancer cell tropism. We profiled its safety and immune response features on day 14 and day 25 of tumor cell inoculation in the 4T1 model. Our data showed that the systemic administration of mHAdLyp.sT resulted in reduced hepatic and systemic toxicity as indicated by serum ALT, LDH and pro-inflammatory cytokine levels when compared with the regular sTGFβRIIFc-expressing adenovirus (Ad.sT). sTGFβRIIFc expression and inhibition of TGFβ-1 were confirmed in serum and/or tumor tissues by ELISA and qRT-PCR, respectively. Our flow analysis revealed that mHAdLyp.sT is effective in increasing anti-tumor CD8$^+$ cells, monocytes/monocytic MDSCs (CD11b$^+$Ly-6C^{hi}Ly-6G$^-$), and M1 macrophages (F4/80$^+$CD11c$^+$) in blood, tumor, or spleen on day 14. We further tested the therapeutic effects of mHAdLyp.sT alone and in combination with immune checkpoint inhibitors (ICIs) in this

model. Although mHAdLyp.sT alone didn't elicit significant changes in primary tumor progression by tumor volume and in tumor weight, histological lung analysis at the endpoint showed significant inhibition of lung metastasis by mHAdLyp.sT ($p<0.0001$ compared to the buffer group). When mHAdLyp.sT was combined with both anti-PD-1 and anti-CTLA-4 antibodies (triple treatment) primary 4T1 tumor growth was significantly inhibited too ($p<0.001$ on day 23). Furthermore, the flow analysis showed that the triple treatment still had a significant increase of $CD8^+$ T cells in blood and spleen at the end of the experiment. Both $CD8^+$ and $CD4^+$ effector or effector memory (TE/EM) subsets ($CD44^+CD62L^-$) in blood were also significantly increased by the triple treatment, suggesting tumor immune stimulation by the triple treatment is sustained for a long time period. Currently, we are further testing optimal doses of mHAdLyp.sT for preclinical studies and exploring more molecular mechanisms behind its interaction with ICIs. We are confident in advancing this new treatment option for mTNBC.

592 Replication Competent Adenovirus-Mediated Cytotoxic and Interleukin-12 Gene Therapy in Prostate Cancer: 36-month Follow-Up Data from a Phase I Clinical Trial

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Introduction: Men with locally recurrent prostate cancer, after definitive radiotherapy, have few therapeutic options. Oncolytic adenovirus-mediated cytotoxic gene therapy is an investigational cancer therapy. Delivery of the suicide gene to the tumor is by direct intratumoral or systemic injection of a viral vector containing the suicide gene. Our approach incorporates yeast cytosine deaminase (yCD) and herpes simplex virus thymidine kinase (HSV-1 TK), to confer sensitivity to 5-fluorocytosine (5-FC) and Valganciclovir (vGCV), respectively. The pro-drugs are converted into active drugs that inhibit DNA damage repair. Here we report the safety of oncolytic adenovirus-mediated suicide gene therapy that incorporates an interleukin-12 (IL12) gene for treatment of recurrent prostate cancer. **Methods:** In this phase I study, a replication-competent adenovirus (Ad5-yCD/*mut*TK_{SR39}rep-hIL-12) expressing yCD/mutTK_{SR39} (yeast cytidine deaminase/mutant S39R HSV-1 thymidine kinase) and human IL-12 (IL12) was injected into tumors of 15 subjects with recurrent prostate cancer (T1c-T2) at escalating doses (1×10^{10}, 3×10^{10}, 1×10^{11}, 3×10^{11}, or 1×10^{12} viral particles). Subjects received 5-FC and vGCV for 7 days. The study endpoint was toxicity through day 30. Experimental endpoints included measurements of serum IL12, interferon gamma (IFNγ), and CXCL10 to assess immune system activation. Peripheral blood mononuclear cells (PBMC) and proliferation markers were analyzed by flow cytometry. **Results and conclusions:** Fifteen patients received Ad5-yCD/*mut*TK_{SR39}rep-hIL-12 and oral 5-FC and vGCV. Approximately 92% of the 115 adverse events observed were grade 1/2 requiring no medical intervention. Ad5-yCD/*mut*TK_{SR39}rep-hIL-12 DNA was detected in the blood of only two patients. Elevated serum IL12, IFNγ, and CXCL10 levels were detected in 57%, 93%, and 79% of subjects, respectively. Serum cytokines demonstrated viral dose dependency, especially apparent in the highest-dose cohorts. Analysis of immune cell populations indicated activation after Ad5-yCD/*mut*TK_{SR39}rep-hIL-12 administration in cohort 5. The study did not detect a significant difference in the PSA doubling time (PSADT) between pre and post treatment by paired Wilcoxon rank test ($p=0.17$). There was no correlation between adenoviral dose and PSADT in each cohort separately or pooled (cohorts1-3 and cohorts 4-5). The study maximum tolerated dose (MTD) was not reached indicating 10^{12} viral particles was safe. This trial confirmed that replication-competent Ad5-IL-12 adenovirus (Ad5-yCD/*mut*TK_{SR39}*rep*-hIL-12) was well tolerated when administered locally to prostate tumors.

593 Targeting Breast Cancer Stem Cells with Mammalian Orthoreovirus (MRV)

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Breast cancer is the second leading cause of cancer deaths among women in the United States. Estrogen receptor positive [ER+] breast cancer has a more favorable prognosis; however, patients can experience recurrence for many years after initial diagnosis. Breast cancer stem cells (BCSC), which are dormant and exist as a minority sub-population (0-5%), drive recurrence, metastasis and resistance to therapies that primarily target rapidly proliferating tumor cells. Current treatments are insufficient to cure metastatic ER+ breast cancer and there are no FDA approved therapeutics that target BCSC. Mammalian orthoreovirus (MRV) is an oncolytic virotherapy tested in clinical trials for many cancer types including metastatic breast cancer. MRV was found to be safe, but efficacy limited as a monotherapy. In our studies we compared several laboratory MRV strains (T1L, R2) to the strain most similar to Reolysin, which is being tested in clinical trials (i.e. T3D). Our studies suggest that T3D is less effective in killing ER+ breast cancer cells and BCSCs. We aimed to generate novel MRV strains with enhanced BCSC-targeting capacity by serially passaging T1L and R2 MRV strains in BCSC enriched 3D tumorsphere cultures. Our preliminary data have shown that serially passaged (SP) MRV strains (T1L SP, R2 SP) are more effective in decreasing MCF-7 paclitaxel resistant (TaxR) cell viability and inhibiting tumorsphere formation compared to the parental (P) strains (T1L P, R2 P). MRV induces cell death by different mechanisms (apoptosis, necroptosis) depending on the cell type. The mechanisms of MRV-induced cell death in BCSC and non-BCSC populations are unknown. We aim to define the effects of MRV-induced death comparing parental vs MRV serially passaged strains on BCSC and non- BCSC cell populations using flow cytometry, and cell death assays. We anticipate these studies will lead to an improved MRV oncolytic virotherapy that will be combined with clinically relevant inhibitors, such as CDK4/6 inhibitors and anti-estrogens, to prevent and treat therapy-resistant ER+ breast cancer.

595 Toca 511/5-FC Treatment Has the Potential to Increase the Therapeutic Efficacy of Anti-PD-1 Antibody in an Immunocompetent Murine Pancreatic Cancer Model

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers and its clinical outcome remains poor, necessitating the development of novel therapeutic strategies. Prodrug activator gene therapy with Toca 511, a tumor-selective retroviral replicating vector encoding an optimized yeast cytosine deaminase (yCD) gene, has demonstrated therapeutic efficacy in a variety of preclinical cancer models and has shown some promise in clinical trials for recurrent high-grade glioma. Toca 511 exerts direct anti-tumor effects through yCD-mediated intratumoral conversion of the prodrug 5-fluorocytosine (5-FC) to the active drug 5-fluorouracil (5-FU). In addition, previous results suggest Toca 511/5-FC suicide gene therapy can also induce anti-tumor immunity. In recent years, immune checkpoint inhibitors (ICIs) were considered as a novel therapy in various cancers, but the therapeutic efficacy of single-agent ICI in clinical trials of advanced PDAC patients was poor. Therefore, various combination therapy with chemotherapy or radiotherapy for PDAC have been investigated to increase sensitivity to ICIs. Here, we first conducted CTL assay and immunohistochemical analysis and investigated the anti-tumor immunity induced by Toca 511/5-FC treatment in an immunocompetent murine bilateral subcutaneous PDAC tumor model. Furthermore, we evaluated the therapeutic effects achieved in combination with anti-programmed cell death protein 1 (PD-1) antibody. Bilateral subcutaneous tumor models were established in C57BL/6J mice by inoculation of the syngeneic murine PDAC cell line Pan02 into both flanks, and ipsilateral flank tumors were initially transduced with Toca 511, while contralateral flank tumors served as untransduced controls. In CTL assays, the 5-FC treatment group demonstrated significantly higher $CD8^+$ T effector cell-mediated cytotoxicity against Pan02 target cells than did the PBS control group. In immunohistochemical analysis, the 5-FC treatment group showed significantly higher T cell infiltration than did the PBS control group. Combination therapy with Toca 511/5-FC treatment and anti-PD-1 antibody achieved significantly stronger tumor growth inhibition against untransduced tumors than Toca 511/5-FC treatment alone. Furthermore, $CD8^+$ or $CD4^+$ T-cell depletion completely eliminated the therapeutic effects of combination therapy. These data showed that $CD8^+$ or $CD4^+$ T-cell-dependent anti-tumor immune responses were important for the therapeutic efficacy of Toca 511/5-FC treatment and combination therapy. These results indicate that Toca 511/5-FC treatment has the potential to increase the therapeutic efficacy of immune checkpoint inhibitors and the combination therapy may be a promising clinical treatment strategy for PDAC.

596 Exploring Novel Viro-Immunotherapies Using "Armed" Oncolytic Adenoviruses and Adoptive T-cell Therapy to Target Pancreatic Ductal Adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related mortalities and is projected to become second by 2030. There is an urgent need for the development of more efficient therapeutics targeting PDAC. Two primary features make PDAC refractory to conventional therapeutics. First, the highly immunosuppressive tumor microenvironment (TME) plays a crucial role in impeding the innate and effector arms of the immune system to prevent anti-tumor responses. Second, desmoplasia causes formation of fibrotic tissue within and around tumor tissue, which creates a physical barrier for infiltration of anti-tumor immune cells and therapeutics. The goal of this project is to develop viro-immunotherapies against PDAC that target the immunosuppressive TME and desmoplasia by utilizing "armed" oncolytic adenoviruses (OAds) and adoptive T-cell therapy (ATCT). I have selected three transgenes to "arm" my OAd constructs: i.) TGF-beta blocker, ii.) interleukin-7 (IL-7), and iii.) interferon gamma (IFN-gamma). Methods: "Armed" replication-deficient adenovirus (Ad) constructs were first generated to isolate transgene function from oncolytic activity. Each transgene was individually cloned into the E1 region of Ad-5 (WT). Viral particles were amplified in 293 cells and purified via ultracentrifugation using a CsCl gradient. Anti-tumor effects were measured using a syngeneic mouse model. C57BL/6 mice were subcutaneously injected with KPC cells (mouse PDAC cell line), and tumors were treated intratumorally with Ad constructs. To measure systemic (abscopal) effects of virus treatment, bilateral tumors were established, and only one side was treated. For IHC staining, tumors were harvested at various time points, fixed in 10% neutral-buffered formalin, sectioned, and stained for immune cell infiltration and fibrosis. Results: Tumor growth was significantly inhibited after treatment with each "armed" Ad construct (Figure 1A). Furthermore, significant tumor infiltration of anti-tumor immune cells was induced upon treatment (Figure 1B) as well as a decrease in fibrosis. Abscopal effects were observed on non-injected tumors after injection of ipsilateral tumors as well as an increase in tumor infiltration of anti-tumor immune cells in untreated tumors (Figure 1C-D). Conclusions: All three "armed" Ad constructs exerted significant anti-tumor effects, and constructs armed with either IFN-gamma or TGF-beta blocker induced significant abscopal effects. These findings have broad implications in the field of pancreatic cancer by shedding light on how the TME can be manipulated to enhance anti-tumor effects to aid in the development of more efficient therapeutics against PDAC. Moreover, therapeutics inducing abscopal effects should be highly valued because most PDAC patients die from metastasis. Future Directions: To obtain a mechanistic understanding of the anti-tumor effects, depletion of immune cells in mice (CD4+ and CD8+ T-cells and NK cells) will be performed prior to virus treatment to see how the absence of these cells affect tumor growth. "Armed" replication-competent constructs will be generated to see how the added effects of oncolysis with transgene activity may enhance anti-

tumor responses. Finally, OAd constructs will be combined with mesothelin-targeted chimeric antigen receptor (CAR) T-cells using xenograft models in SCID mice.

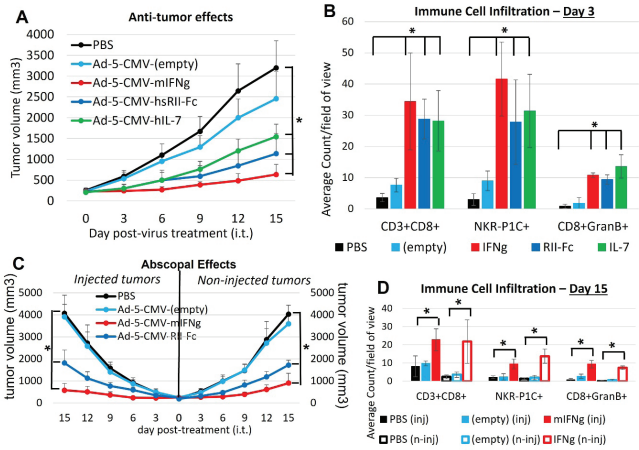

Figure 1.) Anti-tumor effects of "armed" Ad constructs. **A.)** C57BL/6 mice bearing subcutaneous KPC tumors were treated intratumorally with virus constructs at days 0 and 6, and tumor volume was tracked every 3 days. **B.)** Tumors harvested on day 3 were stained for CD3+CD8+ T-cells, NK cells (NKR-P1C+), and activated T-cells (CD8+GranB+). **C.)** Abscopal effects were measured using bilateral subcutaneous tumors and injecting with virus on only one side (day 0 and 6). **D.)** Injected and non-injected tumors were harvested on day 15 and stained as described in **B**. *$P < 0.05$*

597 Targeting Oncolytic Adenoviruses to Cancer Cells Using a Designed Ankyrin Repeat Protein (DARPin) Lipocalin-2 (LCN2) Fusion Protein

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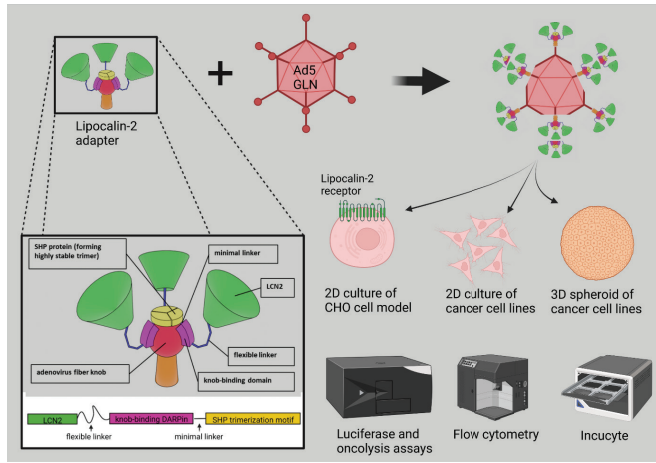
Oncolytic viruses are a promising technology to attack cancer cells and to recruit immune cells to the tumor site. Since the Lipocalin-2 receptor (LCN2R) is expressed on most cancer cells, we used its ligand lipocalin-2 (LCN2) to target oncolytic adenoviruses (Ads) to cancer cells. Therefore, we fused a designed ankyrin repeat protein (DARPin) adapter binding the knob of Ad type 5 (knob5) to LCN2 to retarget the virus towards LCN2R with the aim of analyzing the basic characteristics of this novel targeting approach. The adapter was tested *in vitro* with Chinese Hamster Ovary (CHO) cells stably expressing the LCN2R and on 20 cancer cell lines (CCLs) using an Ad5 vector encoding luciferase and green fluorescent protein (GFP). Luciferase assays with the LCN2 adapter (LA) showed 10-fold higher infection compared to blocking adapter (BA) in CHO cells expressing LCN2R, but also in cells not expressing the LCN2R. Most CCLs showed an increased viral uptake of LA-bound virus compared to BA-bound virus and for five CCLs viral uptake was comparable to unmodified Ad5. Flow cytometry and hexon immunostainings also revealed increased uptake of LA-bound Ads compared with BA-bound Ads in most tested CCLs. Virus spread was studied in 3D cell culture models and nine CCLs showed increased and earlier fluorescence signals for LA-bound virus compared to BA-bound virus. Mechanistically we show that the LCN2 adapter increases viral uptake only in the absence of its ligand Enterobactin (Ent) and independently of iron. Together, we characterized a novel DARPin-based system resulting in enhanced uptake demonstrating potential for future oncolytic virotherapy. A graphical abstract of the applied retargeting strategy is displayed below (Created with BioRender.com).



598 Oncolytic Adenovirus Expressing NIS for the Therapy of Chemoresistant Breast Cancer Stem Cells

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Recurrence of metastatic breast cancer arising from acquired endocrine and chemotherapy resistance remains a health burden for women with luminal estrogen-receptor positive (ER+) breast cancer. Disseminated ER+ tumor cells can remain viable but quiescent for years to decades. A crucial factor contributing to metastatic spread include the maintenance and expansion of breast cancer stem cells (BCSCs). Since BCSCs exhibit enhanced resistance to chemo and endocrine therapies, there is a need for more efficient treatment options. We aim to use Oncolytic adenoviruses (OAd) to target BCSCs. OAd are engineered to selectively replicate in cancer cells leading to the destruction of targeted cancer tissue. Our laboratory has engineered OAd encoding the sodium/iodide symporter (NIS), a protein that mediates uptake of iodine for radiotherapy and imaging. In this OAd, the NIS gene was inserted into the E3 region to allow continual gene expression as the virus replicates under control of the COX2 promoter. To improve infectivity, we have equipped virus with the Ad5/Ad3-modified fiber which allows to CD46 and hDSG2 receptors attachment. We hypothesized that OAd-NIS can target and lyse breast CSCs, resulting in the decrease of local recurrences risk by treating preexisting, clinically undetectable micrometastatic deposits. We demonstrated that our OAd-NIS has enhanced binding and

exhibits superior oncolysis in human breast cancer cells from all breast cancer subtypes, including ER+ luminal cells. We also found OAd-NIS to be selective for breast cancer cells as the virus exhibited minimum oncolytic activity and NIS expression in normal human mammary epithelial cells. The deletion of the Adenovirus Death Protein (ADP) greatly improved the expression of NIS in breast cancer cells, most likely due to greater NIS membrane localization. To confirm the potential of OAd-NIS to target BCSCs population, we employed ER+ paclitaxel-resistant (TaxR) cells and 3D models which have been shown to exhibit stem cell markers (e.g. ALDH+ or $CD44^{hi}/CD24^{lo}$). We have previously shown that TaxR cells generate more tumorspheres compared to the parental, chemosensitive MCF-7 cells, a feature of CSCs to form colonies in serum-free suspension culture. Importantly, the killing effect of OAd-NIS in TaxR cells was improved compared to that in control MCF-7 cells. This correlated well with the increased NIS gene and protein expression in TaxR cells, as shown by qPCR and immunofluorescence staining, respectively. More importantly, in contrast to MCF-7 control, when TaxR cells were co-treated with virus and paclitaxel, we found that OAd-NIS reversed chemoresistance, sensitizing TaxR cells to paclitaxel upon virus infection. Finally, in a tumorsphere assay, we found that OAd-NIS inhibits the formation of tumorspheres in TaxR and MCF-7 3D cultures, and successfully destroys already formed tumorspheres upon infection. This study confirmed the potential of OAd-NIS to target and effectively destroy BCSCs population. Our next steps include evaluation of the stem cell markers in TaxR cells upon OAd-NIS infection and in vivo potential of OAd-NIS to facilitate radioiodine-based imaging and therapy. Our studies also suggest that OAd-NIS could be used as an adjuvant therapy in combination with paclitaxel to treat ER+ breast cancer patients with recurrent metastatic disease. We plan to test this hypothesis using the mouse mammary intraductal injection model, which closely recapitulated human breast cancer progression and will form distant metastasis from ER+ breast cancer cells.

599 Retroviral Replicating Vector-Mediated Prodrug-Activator Gene Therapy Achieved Highly Efficient Tumor Transduction and Antitumor Efficacy in Experimental Human Gastric Cancer

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Retroviral replicating vectors (RRVs) can selectively replicate within tumor cells. RRV can mediate efficient tumor-specific delivery of prodrug activator genes into tumor cells, and subsequent prodrug administration leads to synchronized cell killing of infected tumor cells. Here we evaluated two different RRV derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), which utilize different cellular receptors (PiT-2 and PiT-1, respectively) for viral entry, in human gastric cancer cells. Quantitative RT-PCR showed that expression levels of PiT-2 were higher than PiT-1 in all gastric cancer cell lines tested. Consequently, AMLV encoding the green fluorescent protein gene infected and replicated more efficiently than GALV in most gastric cancer cell lines. In contrast, neither RRV showed efficient infection of normal human fibroblasts. Next, AMLV encoding yeast cytosine deaminase (CD), which converts the prodrug 5-fluorocytosine (5-FC) to the active drug 5-fluorouracil, showed efficient killing of most gastric cancer cells in the presence of 5-FC prodrug compared to GALV. This differential cytotoxicity between AMLV vs. GALV correlated with viral PiT-2 vs. PiT-1 receptor expression level and virus spread efficiency in gastric cancer cells. Moreover, in MKN-74 subcutaneous xenograft models of gastric cancer, AMLV-CD showed significant antitumor effects compared to GALV-CD. Furthermore, in the MKN-74 recurrent tumor model in which 5-FC was discontinued, resumption of 5-FC reduced tumor volume without further administration of RRV. These findings indicate that AMLV-mediated prodrug-activator gene therapy may be beneficial for treating human gastric cancer. Furthermore, custom utilization of alternative RRV strains according to differential expression levels of viral receptors may enable more personalized and effective cancer virotherapy in future studies.

600 Inhibition of c-MYC in Hepatocellular Carcinoma via AAV8 Mediated Gene Transfer

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Inhibiting the key transcription factor MYC has long been a goal due to its central role in human cancers; although, it has yet to be accomplished clinically. Hepatocellular carcinoma (HCC) is the most common liver malignancy in adults and the second most common liver tumor in children and is often associated with MYC deregulation. The mini protein Omomyc has been shown to induce tumor regression in a variety of cancer types by preventing the heterodimerization of c-MYC and it obligate binding partner MAX, thus preventing its transcriptional activation. but questions about in vivo peptide toxicity and efficacy remain. Due to the natural high liver tropism of AAV8, we hypothesized that targeted delivery of Omomyc using the AAV platform will be much more efficacious and limit off target toxicity as opposed to other delivery methodologies. Expression of Omomyc in HCC cell lines *in vitro* by transient transfection and doxycycline induced expression induces an 8-20 fold inhibition of proliferation depending on cell type, including G2 cell cycle arrest (about 50%) in the high c-MYC expressing cell line Hep3B. We have also demonstrated a 2-fold induction of autophagy, measured via mCherry-GFP-LC3 levels in HCC cell lines with a direct correlation to the amount of c-MYC expressed in these cell lines. When combined with conventional therapeutics like the multi-kinase inhibitor sorafenib, Omomyc induces autophagy another 20%. Additionally, Omomyc results in significant levels of cell death (20-60%), in direct correlation with the level of c-MYC protein expression. Our preliminary data suggests that this could be due to induction of autophagic cell death, with mitigation of the effect seen when treated with Bafilomycin A1 or Chloroquine. Treatment of HCC with recombinant AAV8 with vector genomes encoding Omomyc results in a profound cytostatic effect (2-7 fold lower proliferation rate), and very strong cytotoxicity when combined with sorafenib, leading to high levels of cell death in Hep3B cells. We have also demonstrated a potential synergistic effect of combining autophagy inducing compounds like sorafenib and mTOR inhibitors being able

to increase transduction efficiency by approximately 50-60% in our HCC cells lines. Taken together, our data suggest that our novel AAV8 delivery of Omomyc has the ability to inhibit cellular proliferation, induce autophagy, and induce cell death in HCC which may prove to be a novel therapeutic approach for the treatment of HCC, especially when combined with conventional therapeutics.

601 TRAIL Gene Transfection Using Innovative Lipid Vector in Association with Chemotherapy Efficiently Kills Cisplatin-Resistant Ovarian Cancer Cells

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Introduction: Ovarian cancer (OC) is the most lethal gynecologic cancer among women in developed countries. Most of the patients will experience recurrence and succumb to the chemo-resistant disease. Overcoming the resistance to platinum salts represents a major unmet need. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is capable of inducing apoptosis primarily in tumor cells upon ligation with its death receptors (DR). Its pro-apoptotic effect has been well investigated. However, the application of recombinant forms of TRAIL and agonistic antibodies of its receptors have provided disappointing results in clinical trials due to short half-life *in vivo*, potential hepatotoxicity and poor cancer-targeting ability. Thus, TRAIL gene therapy has been developed to overcome these limitations. Here we described the killing effect of TRAIL gene, transfected by our innovative bioinsprired lipid vector BSV163/DOPE (**Figure 1**), in association with cisplatin to treat ovarian cancer cell lines and its cisplatin-resistant analogs. **Methods:** Caov3 and OVCAR3 cell lines were selected to represent high-grade serous ovarian cancer. Cisplatin-resistant Caov3 (CR-Caov3) cells were achieved by culturing Caov3 cells in the presence of cisplatin over 6 months. Luciferase-encoding plasmid (pGM144, 3759 bp) was used as a transfection control plasmid for TRAIL-encoding plasmid (pVAX2-TRAIL, 3771 bp). The lipid vector BSV163/DOPE was formulated and used to encapsulate and transfect various plasmids. Transfection properties were determined by the luciferase assay and RT-qPCR. Cell viability following different treatments was evaluated with MTT assay. Mechanistic studies were also carried out. **Results:** The cisplatin sensitivity of various cell lines was evaluated by MTT assay. CR-Caov3 cells were more resistant to the killing effect of cisplatin than the parental line at various concentrations after 48 or 72 hours incubation. Luciferase activity was determined after cellular transfection using pGM144 complexed by BSV163/DOPE at different charge ratios (CR 2-4) and CR4 was considered the most effective. Using CR4, we transfected both cisplatin-sensitive and -resistant ovarian cancer cells with BSV163/DOPE/pVAX2-TRAIL and verified the expression of TRAIL mRNA. The apoptosis rate in cells treated with the combinatory treatment (TRAIL transfection + cisplatin) was higher in comparison with each treatment separated. In CR-Caov3 cells, the apoptosis rate induced by the combined treatment, TRAIL transfection, or cisplatin were 40%, 20% and 10% respectively. These results demonstrated a substantial interest in using BSV163/DOPE as a gene delivery system in ovarian cancer gene therapy and encourage the use of TRAIL transgene to fight cisplatin resistance.

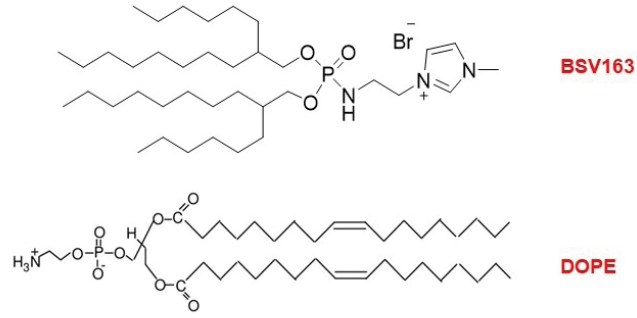

Figure 1. The structure of cationic lipid vector BSV163 and neutral helper lipid DOPE

602 An All-Prime Editing One-Step Approach for Non-Viral Generation of a Multiplex-Edited Allogeneic CAR-T Cell Product

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Despite the success of CAR-T cell therapy in the treatment of a subset of hematological malignancies, the autologous nature of commercially available CAR-T products and limited clinical efficacy against solid tumors have delayed broad application of this therapy. In particular, manufacturing autologous CAR-T therapies is costly and time-consuming, and the rate of manufacturing failure is high. To overcome these limitations, targeted nucleases have been used to disrupt genes involved in alloreactivity and T cell regulation, with the goal of creating an allogeneic 'off the shelf' CAR-T cell product with enhanced function. The use of nucleases such as CRISPR-Cas9 to generate a multiplex edited allogeneic CAR-T cell product may be limited due to the risk of chromosomal rearrangements and genotoxicity that can occur when double-strand breaks (DSBs) are induced at multiple loci simultaneously. Alternatively, base editors have been used for targeted gene disruption without the induction of DSBs via single nucleotide conversion to disrupt a splice site or introduce a single premature stop (pmSTOP) codon. However, this approach is limited to transition mutations and cannot be used to integrate large genetic cargo, which is required to generate CAR-T cells. Prime Editing (PE) can be used for precise and programmable gene disruption via multiple strategies, including introduction of multiple pmSTOP codons, splice site disruption, frameshift mutations, or deletion of large segments of regulatory or coding sequence. Further, Prime-Assisted Site-Specific Integrase Gene

Editing (PASSIGE) can be used to precisely and flexibly integrate large genetic cargo at a specific locus. Together, PE-mediated gene knock out and PASSIGE can be tailored to generate a more broadly applicable, potentially safer, and more effective CAR-T cell product. To evaluate the efficacy of an all-PE non-viral approach to CAR-T cell generation, PASSIGE and PE mediated gene knockout were used to generate CD19-CAR-T cells. PE precisely knocked out the endogenous T cell receptor alpha constant (*TRAC*) locus in up to >90% of human T cells. Disruption of the endogenous TCR is required to prevent graft-versus-host disease (GvHD) in the context of an allogeneic T cell product. Co-delivery of a non-viral DNA donor template with PASSIGE editing components resulted in targeted integration of a 3.5 kb CD19-CAR transgene expression cassette at the *TRAC* locus in >60% of the T cells, with no observed impact on T cell viability, phenotype, or functionality. CD19 CAR-T cells generated using PASSIGE show potent anti-tumor activity and cytokine production in response to $CD19^+$ tumor cell lines. Further experiments demonstrated efficient multiplex PE at up to three target sites simultaneously and PASSIGE mediated integration of the CD19-CAR at the *TRAC* locus. These results show that a PE platform can be used to generate multiplex edited CAR-T cells without the need for viral vectors and without causing DSBs. This modular, one-step, nonviral delivery Prime Editing platform expands the applicability of T cell therapies for the treatment of tumors and immune diseases.

603 Safety-Switch Engineered Anti-CD117 CAR T Cells Eradicate Human Acute Myeloid Leukemia and Hematopoietic Stem Cells

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Acute Myeloid Leukemia (AML) derives from the acquisition of mutations in hematopoietic stem and progenitor cells (HSPCs), which gives rise to a rare population of malignant leukemia-initiating cells (LIC). As AML-LICs are a source of disease relapse after treatment, their eradication is essential for the cure of AML. A main issue in the field is the sharing of cell-of-origin surface antigens between HSPCs and AML-LICs, baring the consequence of healthy HSPC depletion and subsequent severe myeloablation. Depletion of CD117+ cells can nevertheless facilitate bone marrow niche clearance and subsequent allogeneic HSC transplantation (HSCT) in absence of non-genotoxic preconditioning. However, this concept implies early termination of chimeric antigen receptor (CAR) T-cell activity to prevent subsequent graft rejection. In the current study, we therefore exploit several non-viral technologies for the generation of engineered T cells expressing anti-CD117 CAR molecules as well as safety-switches to pre-clinically test their efficacy and termination. We showed that electroporation of an IVT mRNA encoding an anti-CD117 CAR is feasible to generate functional T cells that temporarily express CD117 CAR molecules, exhibit transient cytotoxicity, and *in vivo* activity against CD117+ cells when infused in at least two high doses into mice. Alternatively, stable expression of anti-CD117 CAR and the iC9 suicide gene in T cells by using an integrating sleeping beauty (SB) vector allowed for fit CAR T-cell products associated with high proportion of T memory stem cells and low levels of exhaustion markers. SB anti-CD117 CAR T cells showed potent cytotoxic activity *in vitro* and underwent apoptosis when iC9 transgenes were activated. Anti-CD117 CAR T cells engineered with the SB vector also showed anti-leukemic activity in a human tumor xenograft model and completely depleted healthy HSPC in immunodeficient mice reconstituted with a human hematopoiesis. Our data indicate that CAR T cells have to persist two weeks for achieving complete HSPC depletion, which may also lead to durable leukemia remission. Furthermore, CAR T cells were terminated *in vivo* by combining activation of iC9 and Antithymocyte Globulin (ATG) administration, which would allow for CAR T-cell depletion before allo-HSCT. Notably, we observed no difference in activity when CAR T cells produced by a non-viral approach with the SB vector were compared with cells engineered with LV vectors. Taken together, our results indicate that non-viral engineering with SB can produce functional CAR T cells even with complex vectors that include safety switch delivery. Anti-CD117 CAR T cells can lead to potent anti-tumor activity and complete eradication of HSPCs, envisioning its application prior to allo-HSCT in early clinical trials of patients with high-risk AML or Myelodysplastic Syndrome.

604 Expanding the Scope of Multiplex Engineering for Superior Generation of Efficient CAR T-cells

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CAR T-cell therapies have revolutionized the way we can treat hematological malignancies. In the meanwhile, the presence of additional physiological and biological barriers imposed by the microenvironment has limited the ability to target solid tumors. In recent years, advances in genomic-based cellular engineering are bringing us a step closer to conquer solid tumors. This glimpse of success also demonstrated that we need to be able to creatively modify and equip CAR T-cells to target these tumors. A caveat to this is that increasing the number of genetic modifications at the cellular level could compromise the level of editing efficiency as well as lead to genomic toxicities. Here, we show that we can use our state-of-the-art TALEN® technology to precisely edit up to four loci simultaneously while delivering several additional payloads to increase the efficacy and persistence of CAR T-cells. We take it a step further and use a curated combination of genome engineering technologies including

TALE base editors to increase the efficiency of multiplexed gene editing while protecting genomic integrity. By carefully choosing a range of gene and cell engineering approaches, we can develop CAR T-cells focused on unmet medical needs with a high level of efficiency for gene editing and targeted-integration. We also show that this high efficiency in TALEN®-mediated gene-edition does not lead to increased gene integrations at undesired loci. Moreover, we show that multiplexed engineering does not compromise CAR T-cell function, which in turn can be enhanced and display improved anti-tumor activity. Thus, multiplexed engineering at superior efficiency rates while preserving genomic integrity has the potential to generate highly functional CAR T-cells to advance in the fight against solid tumors.

605 Patient Based Selection of Receptors Driving Chemokine-Mediated Homing Enhances B7H3.CAR T Cell Efficacy Against Pediatric Osteosarcoma

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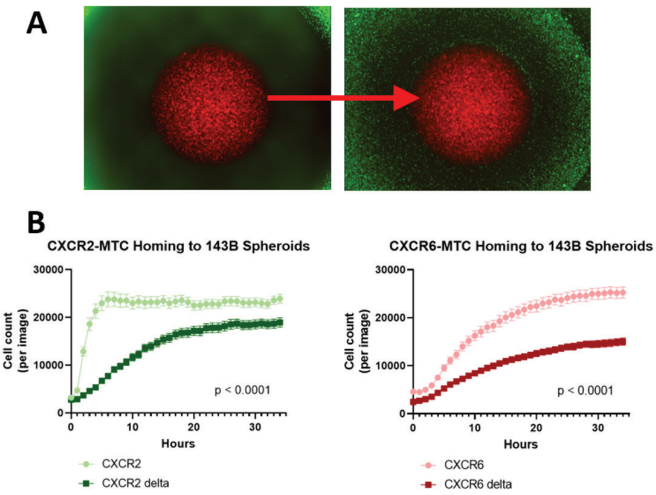
Background: Despite significant preclinical promise, clinical efficacy of CAR T cells against osteosarcoma (OS) has been limited. One strategy to improve their efficacy may be to drive chemokine-mediated homing of CAR T cells to tumor sites. We sought to determine the primary chemokines secreted by OS and evaluate efficacy of CAR T cells expressing the cognate receptors. **Methods/Results**: We developed a patient-based pipeline to identify chemokines secreted by pediatric solid tumors. RNA-seq data for 50 known chemokines was obtained from 456 tumor samples in the CSTN network. Simultaneously, chemokine secretion was quantified in media in which surgical specimens post resection are transported at our institution (n = 8). We identified 7 chemokines produced by OS (IL8, CXCL10, CXCL12, CXCL16, RANTES, CCL18, CCL21) and assessed cognate receptors for expression on donor T cells (n = 5). CXCR2 (0.34%) and CXCR6 (3.3%), cognate receptors for IL8 and CXCL16, had minimal endogenous expression. We produced lentiviral vectors expressing CXCR2, CXCR6, or a control receptor and transduced donor T cells to assess quality and chemotaxis (n = 5). CXCR2- and CXCR6-T cells had similar phenotypes to controls. In transwell assays, CXCR2- and CXCR6-T cells exhibited dose-dependent migration towards rhIL8 ($p < 0.0001$) and rhCXCL16 ($p < 0.0001$). In a novel 3D spheroid homing assay, CXCR2- and CXCR6-T cells demonstrated improved homing towards 143B OS spheroids compared to controls ($p < 0.001$), with CXCR2-T cells having faster homing kinetics than CXCR6-T cells and controls, but similar plateau (**Fig 1**). To assess *in vivo* homing, 143B tumors were surgically implanted in the right tibia of NSG mice, and IV injected ffluc-(+) CXCR-T cells were tracked by bioluminescence. CXCR6-T cells exhibited enhanced homing to tumors compared to CXCR2-T cells and controls ($p < 0.0001$, n = 5/group). We next evaluated chemotactic and antitumor activity of CXCR2- and CXCR6.B7-H3.CAR T cells. CXCR2 or CXCR6 expression in B7-H3.CAR T cells did not change their phenotype, but increased IFNγ production (n = 6; $p < 0.01$) in 143B coculture assays with no effect on IL2 production. Homing of CXCR.B7-H3.CAR T cells in 3D spheroid assays mirrored that seen with CXCR-T cells, which was enhanced compared to B7-H3.CAR T cells (n = 5, $p < 0.001$). CXCR2.B7-H3.CAR T cells also demonstrated faster killing in 3D spheroid culture compared to CXCR6.B7-H3.CAR and B7-H3.CAR T cells ($p < 0.001$). *In vivo,* CXCR2- and CXCR6.B7-H3.CAR T cells demonstrated earlier tumor-specific homing and expansion (n = 5/group; $p < 0.05$ at days 9, 10, 17 - 27) with less non-specific expansion compared to B7-H3.CAR T cells. Finally, 143B tumor-bearing mice injected IV with CXCR2- or CXCR6.B7-H3.CAR T cells survived longer than mice treated with B7-H3.CAR T cells (n = 5-10/group; $p < 0.001$). **Conclusions**: Patient-based determination of chemokines secreted by pediatric OS identifies robust targets for chemokine receptor modification of CAR T cells. CXCR2 and CXCR6 both enhance homing of B7-H3.CAR T cells, with improved antitumor activity and evidence of reduced non-specific expansion *in vivo*. Optimization of these receptors with additional CARs active in OS is warranted.



606 *In Vivo* Genetic Medicine Designed to Simultaneously Engineer Innate and Adaptive Immune Cells to Express Chimeric Antigen Receptors and Generate Durable, Anti-Cancer Immune Responses

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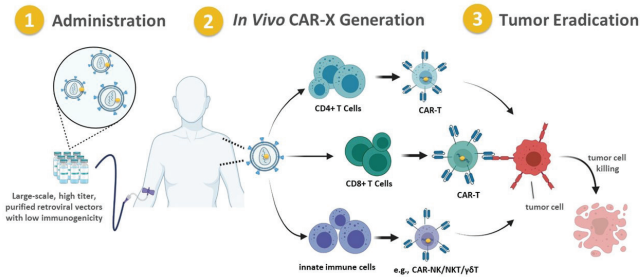
The gene therapy field has advanced tremendously since the first gene therapy patient was functionally cured in 1990 with a T cell therapy modified with a gammaretroviral vector (gRV). The safety and tolerability of gRV is supported by clinical trials involving direct in vivo administration in over 600 patients and in trials involving ex vivo use in 1,000's of patients without a single incident of integration-related toxicity spanning more than 2 decades. These advances include the market-leading CAR-T product, axicabtagene ciloleucel, which is made using gRV vectors to achieve stable CAR expression. Despite durable clinical efficacy in about 40% of patients with relapsed or refractory

lymphoma, issues related to safety and costs have hampered patient access in the community setting where most lymphoma patients are treated. Meanwhile, in vivo engineering of circulating immune cells was seen in several clinical trials involving intravenous administration of gRV manufactured with high titer, high purity, and low immunogenicity at commercial GMP scale and at a comparatively low cost. Preferential gene delivery to circulating immune cells was attributed to a short half-life and requirement for dividing cells. Building on these advances we are developing an in vivo CAR product candidate, ABIN-19, which simultaneously engineers innate and adaptive immune cells and may overcome the challenges with autologous and allogeneic ex vivo cell therapies (see figure below).



We report on the preclinical development of ABIN-19, which is designed to engineer circulating innate and adaptive immune cell subsets to express CAR. High titer, high purity gRV encoding a CD19-CAR was intravenously delivered to CD19+ syngeneic lymphoma or humanized-NSG rodent models. In the syngeneic models, after intravenous delivery of vector encoding CD19 CAR, we saw simultaneous engineering of circulating innate and adaptive immune cells including natural killer (NK) cells, alpha/beta T cells (primarily effector and central memory subsets), monocytes, and natural killer T cells. Corresponding with antitumor effects, NK cells showed markers of activation including upregulation of CD69 and KLRG1. Further, alpha/beta T cells upregulated CD25, an activation marker, and expansion of central memory subsets was observed. Corresponding with T cell activation was emigration of innate and adaptive immune cells out of the periphery to sites of lymphoma. In non-tumor bearing humanized NSG models, injection of vector encoding CD19 CAR resulted in T cell activation and subsequent CD19+ B cell aplasia, a phenomenon also reported in ex vivo CAR T models. In vivo CAR products, such as ABIN-19, that engineer innate and adaptive immune cells in situ, have the potential to achieve a differentiated efficacy and safety profile and warrant clinical investigation.

607 Innovative 4th Generation Lentiviral Vectors to Enable *In Vivo* CAR T-cell Generation

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CAR T-cell therapy is an important and exciting treatment modality with several approved products. While significant clinical benefit has been observed in patients there are several challenges with autologous *ex vivo* CAR T therapies: cell processing is complex, can take time and is expensive. Furthermore, the limited availability of appropriate GMP manufacturing facilities and clinical sites, restricts both the number of patients that can be treated and the geographical locations in which treatment can be offered. In addition, CAR T-cell fitness is impacted during manufacture which can impair clinical success. Generation of CAR T-cells within the patient themselves (*in vivo*) would bypass the need for *ex vivo* cell manufacture leading to substantial cost reduction, avoid cell manufacturing failures and delays and therefore treatment would be more widely accessible. Here, we present findings on several technical developments and innovations in our lentiviral vector platform that have the potential to improve the safety and efficiency of *in vivo* CAR T-cell generation. Firstly, we utilise a transgene repression system in the producer cell which prevents CAR incorporation into the vector particle which therefore avoids the unwanted transduction of tumour cells, which would otherwise lead to masking of the antigen and loss of tumour control. In addition, this approach could reduce possible immune reactions either to CAR on viral particles or on transduced cells. During *ex vivo* CAR T-cell generation, viral envelope proteins with a broad cellular tropism, such as VSVG, are routinely used. However, the safety and efficacy of *in vivo* CAR therapy will require a toolkit of vectors that target specific immune cell subsets. We therefore investigated modifications to viral envelope proteins that would alter the cellular tropism and re-target vectors to specifically transduce T-cells. We have demonstrated *in vitro* that molecular retargeting of lentiviral vectors can drive highly selective transduction of T cells and generate functional CAR T-cells. Finally, we combined several of these innovations and tested systemic injection of CAR lentiviral vectors in mice with a humanised immune system. We show the vectors are well tolerated, drive the generation and expansion of CAR immune cells in the blood, and can also lead to the expected B cell aplasia. In conclusion, *in vivo* generation of CAR cells by the systemic injection of a lentiviral vector has the potential to dramatically widen the availability and accessibility of treatment. We report a number of technical developments and innovations that address key challenges to developing a safe and effective *in vivo* CAR cell therapy which, together with Oxford Biomedica's ability to produce high quantities and high quality of commercial grade vector, will form the basis for new *in vivo* CAR-T products with which to treat patients for oncology and autoimmune indications.

608 RESET: A Novel TCR Coupled Antigen Receptor Displaying Superior Targeting Sensitivity and Pharmacologically Controlled Anti-Tumor Activity

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Chimeric antigen receptor (CAR) T cells are effective therapies for hematological malignancies, where target antigens are exclusively yet robustly expressed on the tumor and cells with low toxicity risks (e.g., B cells). For other tumors, available target antigens often lack ideal expression, necessitating improvements in targeting sensitivity, potency, control, and/or exhaustion mitigation to achieve robust anti-tumor efficacy. Endogenous T cell receptor (TCR) activation is acutely sensitive to low target abundance and naturally propagates effector and long-term memory functions, suggesting that mimicking TCR activation may provide a solution. Here, we describe a novel receptor architecture called RESET (rapamycin-enabled, switchable endogenous TCR) that combines: (i) the antibody-based targeting of CARs; (ii) the drug-regulated activity of the DARIC technology (a "drug on" CAR T system); and (iii) the natural signaling proficiency and inherent sensitivity of TCRs. The RESET receptor comprises two functional units that enable toggling of RESET T cells between inert and activated states through rapamycin-induced dimerization. Specifically, RESET appends the FKBP-rapamycin binding protein (FRB) to CD3ε, which naturally integrates into the endogenous TCR-CD3 complex. A second unit is expressed on the T cell surface that includes a tumor targeting antibody linked to FK506-binding protein (FKBP). Dimerization of FRB and FKBP occurs with the addition of rapamycin, linking tumor targeting to the endogenous TCR and driving T cell activation in an antigen-dependent and rapamycin-regulated manner. Complete dependence of RESET function on TCR integration was confirmed by transducing RESET into T cells edited to eliminate TCRα expression. Surface trafficking of the FRB-CD3e polypeptide was lost in the absence of the TCR, concomitantly ablating RESET T cell function.Next, we explored the antigen-dependent behavior of RESET T cells. Despite requiring rapamycin for full activation, standard and drug-regulated CAR T cells can display tonic signaling (e.g., cytokine release) that prevents them from achieving a fully inert state when rapamycin and/or antigen are absent. In contrast, TCR coupled RESET T cells do not induce tonic signaling, indicating that the "off" state of this architecture is more complete. CD33-targeted RESET T cells (RESET33) are also more potently reactive to CD33+ tumor cells after rapamycin dimerization *in vitro*, secreting ~3x more cytokine than CD33-targeted regulated CAR T cells when challenged with low antigen density tumor cells. This enhancement was also observed in preclinical NSG mouse models *in vivo*, where RESET33 T cells more deeply controlled systemic CD33+ AML tumor models, providing a significant survival benefit in comparison to DARIC33 T cells. Our data suggest that RESET T cells outperform standard drug-regulated, second-generation CAR T cells and show hallmarks of TCR activation that suggest targeting fidelity comparable to native T cell responses. Pharmacological control serves to broaden the scope of targetable antigens and may mitigate T cell exhaustion caused by continuous antigen exposure. The convergence of drug regulated antigen targeting and natural immune receptor signaling may better replicate the kinetics and physiology of T cell responses, resulting in more successful immunotherapies. Taken together, these data support the clinical evaluation of RESET in a target indication such as AML, where "drug-on" targeting with TCR signaling sensitivity may enable more robust anti-tumor activity, while the more complete "drug-off" state allows for myeloid reconstitution and provides relief from T cell exhaustion.

609 Cell and Molecular Optimization Overcome Barriers to Enable CRISPRa-Driven Genome-Wide Gain-of-Function Screens in Primary CAR T Cells

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Chimeric Antigen Receptor (CAR) T cell therapy has revolutionized the treatment of B cell malignancies and translating this success to other cancers remains an ongoing clinical objective. Next-generation T cell products in development today aim to genetically modulate many facets of cell behavior, and gene-nominating platforms have provided a useful framework for prioritization. Among competing screening approaches, CRISPR activation (CRISPRa) technology permits gain-of-function (GoF) gene surveys at genome-wide scale, but routine implementation in primary T cells has been stymied by high cell requirements (~10^8) and poor dCas9 expression. Here, we describe a novel cell manufacturing schema using an all-in-one transposon-based gene delivery system coupled with CAR-restricted cell expansion to generate yields of primary T cells bearing CAR and CRISPRa transgenes, well above the threshold needed for genome-scale screening. Long-term GoF potency is achieved via vector optimization to prevent CRISPRa gene silencing (>90%$^+$); library representation is preserved by delaying guide RNA transfer until late in primary manufacturing, thus preventing bottlenecking and premature candidate pruning. CRISPRa-CAR T cells manufactured via this pipeline retain potent on-target gene-overexpression (>85% target$^+$) across varied cell subsets (e.g. Tim-3$^+$/Lag3$^+$) and timescales (>14 days). When deployed to survival-based genome-wide selection landscapes, CRISPRa-CAR pools can successfully nominate endogenous genes capable of enhancing both CAR T persistence and cytokine-free survival. This system will have broad utility for therapy-enhancing gene discovery.

610 Effective Targeting of Gastric Cancer Peritoneal Metastases with Cytokine-Endowed TAG72-Directed CAR T Cells

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Gastric cancer (GC) with peritoneal metastases (PM) is fatal within 8 months of diagnosis. Molecular heterogeneity of GC and poor immunogenicity of peritoneal immune tumor microenvironment

(TME) limit the benefits of existing immunotherapeutic strategies that include trastuzumab (anti-HER2), ramucirumab (anti-VEGF), pembrolizumab, and nivolumab (anti-PD-1). To address the immunosuppressive and immunologically "cold" nature of the peritoneal TME, we investigated a novel chimeric antigen tumor-associated glycoprotein 72 (TAG72) and interleukin-12 (IL-12) combination in GCPM. From 28 patients, surgically resected primary gastric tumors identified to have positive TAG72 expression in >90% of the specimens stained with anti-TAG72 antibody. Encouragingly, 45% showed "strong" TAG72 expression supporting TAG72-CAR T cells as an attractive therapeutic target in GC. A TAG72-CAR T cell (currently in a phase 1 trial for patients with ovarian cancer) cocultured with malignant ascites obtained from GC patients with PM effectively target TAG72+ peritoneal GC cells. We further studied this phenomenon using SNU-16, a human gastric cancer cell-line derived from ascites with strong TAG72 expression. With this model, we show that TAG72-CAR T cells can effectively and consistently target SNU-16 leading to reduced tumor burden and prolonged survival in NSG mice. Additionally, our newly engineered TAG72-CAR T cells with a membrane-bound version of the IL-12 (mbIL12) cytokine significantly enhanced anti-tumor efficacy of TAG72-CAR T cells leading to more durable *in vivo* therapeutic responses. A novel immunotherapeutic combination using autologous mbIL12-engineered TAG72-CAR T cell therapy is a promising strategy to help overcome the therapeutic failures in GCPM patients.

611 Identifying Anti-Tumor Lymphocytes by Immune Fingerprinting Solid Tumors

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Background: Tumor infiltrating lymphocytes (TILs) are all lymphocytic $CD3^+$ cells that have interacted with and invaded the tumor tissue. These cells might display a cytotoxic antitumor activity and therefore be crucial for promoting anti-tumor response or on the other hand dampened it. Regardless of the roll they play, overall assessment of TILs profile and immune status of the tumor microenvironment has its practical value when assessing tumor mutational burden, effectiveness anti-tumor therapy or when designing and monitor T-cell based therapy. Large numbers of formalin-fixed paraffin embedded tumor tissues Worldwide are valuable source of material for TILs analysis. However, time during which tissue was fixed and stored in this way has a detrimental effect on the quality of the obtained RNA material and it is a limiting factor in obtaining TILs repertoire. **Methods:** We performed an immune repertoire profiling analysis of four different solid tumor types, namely: ovary, colon, lung, and breast, all being formalin fixed, paraffin embedded and stored for different periods of time (ranging from 15 months up to 8 years). All samples were stored at room temperature. In brief, histological tumor samples were examined for the presence of T cell. Following, consecutive sections of 10um in diameter were made and RNA was isolated. Assessment of the RNA quality was determined based on DV200 parameter. Following, the presence of T cell RNA material was determined by quantifying the expression of CD247. To assess immune status of the tumor, we performed whole-tissue RNA sequencing. **Results:** Pathological tissue assessment in these four tumors was varying between 20 and 100%, having the smallest portion in the breast carcinoma and largest in the ovary adenocarcinoma. No fibrosis and no necrosis were noticed in any of the samples. T-cell infiltrations were approximately occupying from 3% to 15% of the tumor tissue. Isolated RNA material was of the sufficient quality for the immune repertoire profiling as the DV200 value in all sample was higher than 50%. TCR repertoire profile was successfully obtained from ovary and colon adenocarcinoma. Clonal analysis was performed utilizing TCR beta reads and particular clones were determined using sequence of the CDR3 domain. Prolonged storage increased singletons and unmapped reads. Furthermore, RNA sequencing was successfully performed in all tumor samples regardless of the storing time providing a full immune profile of the tumor microenvironment. **Conclusion:** Our results indicate that duration of the storage significantly affect robustness of the obtained TILs repertoires.

612 A Novel TGFβ Switch Receptor Drives Robust MAGE-A4 TCR Anti-Tumor Activity with a Favorable Safety Profile

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Adoptive T cell therapies have transformed clinical practice in advanced B cell cancers but have yet to achieve similar responses in other hematological or solid tumors. Two dominant factors limit robust efficacy in these settings: the rarity of antigens with tumor specific, high density, homogenous expression; and the immunosuppressive milieu within solid tumors. Thus, we sought to develop a T cell therapy targeted to a tumor antigen with demonstrated clinical efficacy and safety and further layered with technology that not only mitigates but exploits immunosuppression to enhance anti-tumor responses. MAGE-A4 is a cancer/testis antigen with robust intracellular expression in several solid tumors yet is undetectable in healthy tissues. Here we describe a TCR targeting an HLA-A*02:01 presented MAGE-A4 peptide engineered to achieve obligate TCR α-chain and β-chain heterodimerization, improving transgenic MAGE-A4 TCR expression and minimizing mispairing with endogenous TCR chains. In vitro safety studies confirmed the specificity of this TCR, revealing a limited set of off-target peptides which were derisked in overexpression studies, and no reactivity to a panel of normal cells. Solid tumors frequently secrete TGFβ, a pleiotropic cytokine that suppresses T cell responses. Pharmacological inhibition of TGFβ is challenging due to the existence of multiple isoforms with distinct functions, toxicity due to TGFβ function in healthy tissues, and the complexities of inactive TGFβ secretion and activation. Instead, we aimed to counteract the immunosuppressive effects of TGFβ within engineered T cells. We developed a two-component, bifunctional chimeric TGFβ-IL12 switch receptor (CTBR12) whose polypeptides: (i) pair with the endogenous TGFβ receptor chains and, upon TGFβ binding, block downstream suppressive signaling in a dominant negative manner; and (ii) when paired together, convert TGFβ binding into pro-inflammatory signaling events that enhance anti-tumor function. T cells transduced with a

multi-cistronic lentiviral vector co-expressing the pairing-enhanced MAGE-A4 TCR and CTBR12 demonstrated robust antigen-dependent responses that were significantly enhanced upon exposure to TGFβ, whereas MAGE-A4 TCR controls lacking CTBR12 were substantially suppressed. We observed biochemical signatures and functional hallmarks of concurrent TGFβ receptor inactivation and IL12 receptor pathway activation, which were also fully dependent on the presence of TGFβ. CTBR12 co-expression did not alter the safety profile of the MAGE-A4 TCR. In two murine xenograft tumor models with abundant TGFβ, T cells co-expressing the MAGE-A4 TCR and CTBR12 both controlled tumors at T cell doses that elicited only minimal responses without CTBR12 co-expression and exhibited superior durability of response overall. Pharmacokinetic analysis demonstrated 1.5-2-fold enhanced peak T cell expansion in the MAGE-A4 + CTBR12 cohort relative to MAGE-A4 TCR alone, and importantly both cohorts showed similar longitudinal contraction of T cells, demonstrating that the physiological regulation of T cell dynamics is not grossly disturbed by CTBR12. Taken together, the combination of a highly active TCR with a strong specificity profile and a synthetic receptor that leverages an immunosuppressive cytokine to deliver a pro-inflammatory signal has the potential to safely enhance T cell potency in otherwise intractable solid tumors. Our data support an upcoming clinical trial to evaluate the safety and efficacy of autologous T cells co-expressing the pairing enhanced MAGE-A4 TCR with CTBR12 in solid tumors.

613 A New Anti GD2 CAR T Cell Construction Containing GITRL Coestimulatory Molecule Combines Persistent Lysing Capacity and IFNy Secretion Against Glioblastoma Cells

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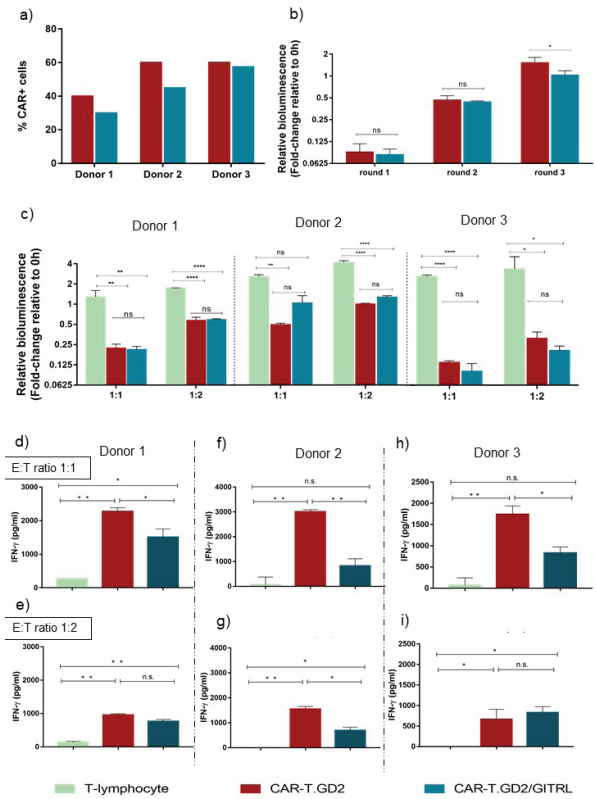
The adoptive transfer of T cells expressing chimeric antigen receptors (CAR) has resulted in the complete B-cell malignancies remission rates. However, the therapeutic efficacy of CAR-T cells is still low or non-existent against solid tumors, which constitute the vast majority of neoplasms. One of the main reasons for this failure is the presence of ligands and immunosuppressive cells in the tumor microenvironment. Also, full activation of T cells requires the engagement of costimulatory molecules whose expression is temporally segregated and whose nature of biochemical signals are complementary. This is the case of CD28, expressed constitutively, and GITR, expressed right after the initial activation of T cells. Therefore, our hypothesis is that the combined expression of a CAR containing the costimulatory domain CD28 and the GITR ligand (GITRL) will potentiate the antineoplastic action of CAR-T cells and suppress the action of regulatory T cells in the tumor microenvironment, thus possibly reducing local immunosuppression. Then, the aim of this study was to evaluate in vitro the therapeutic efficiency of anti-GD2 CAR-T cells coexpressing GITRL using as a model a CAR against the ganglioside GD2, which is highly immunogenic and expressed in glioblastoma. To test our hypothesis, we generated two lentiviral vectors to express CAR anti-GD2 (CAR.GD2) and another one coexpressing CAR anti-GD2 and GITRL (CAR.GD2/GITRL). Next, we used these lentiviral particles to transduce human T cells isolated from PBMC samples of three different donors, and we achieved CAR positive cells frequency in 40-60% of the cells transduced with CAR.GD2 vector and 30-58% for CAR.GD2/GITRL vector. To verify the functional activity of these CAR-T cells, we performed a coculture against luciferase-expressing GD2+ T98G glioblastoma cells and GD2- HCT-166 colon carcinoma cells. We tested two effector:target ratios (E:T). After coculture, we observed a potent cytotoxic activity for both CAR vectors, with around 90% of T98G cell lysis, while sparing HCT-116 cancer cells, demonstrating GD2-restricted cytotoxic activity. After that, we asked whether these CAR-T cells could keep their lysis capacity after additional rechallenges of coculture. Indeed, the cytotoxic effect was persistent after two additional coculture rounds and the CAR-T.GD2/GITRL was able to retract the tumor cell proliferation during the third round better than CAR.GD2 cells. Additionally, we detected high levels of IFNy in the coculture supernantants samples of both CAR-T cells demonstrating that tumor cell killing was associated with secretion of IFNy. Therefore, the potential results of this project will foster the development of a new advanced cellular immunotherapy strategy for the treatment of solid GD2+ neoplasms. Figure subtitle: a) CAR+ cells frequency between T cells of three donors transduced with vectors CAR.GD2 and CAR.GD2/GITRL; b) three rounds of coculture to demonstrate the persistent lysis capacity; c) coculture assay to evaluate the cytotoxic effect against glioblastoma cells in two E:T ratios; d-i) IFNy secretion in coculture supernantants.



614 Antigen Binding Domain Influences CD47-CAR T Cell Function in Both Constitutively Active and Inducible Systems

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Chimeric antigen receptor (CAR) T cell therapy has shown considerable success against hematological malignancies but has yet to exhibit the same success against solid tumors. Several roadblocks must be overcome to yield successful CAR T cell therapies for solid tumors, including limited T cell persistence, the immunosuppressive tumor microenvironment (TME), and tumor heterogeneity/antigen escape. One way to target solid tumors and potentially overcome some immune suppression within the TME is by targeting CD47. CD47 is a 'macrophage don't eat me' signal that is highly expressed by multiple solid tumors to evade immune-mediated elimination. As CD47 is expressed at lower levels on normal tissues, inducible CD47-CAR T cells activated only within the TME may achieve antitumor activity with limited 'on-target, off-cancer' toxicity. We previously demonstrated that high-affinity SIRPα receptor variant based CD47-CAR (CV1-CAR) T cells downregulate CD47 on their cell surface to evade fratricide and enable antitumor activity in vitro. However, loss of surface CD47 prevents CAR T cell persistence in vivo due to macrophage-mediated elimination. Here we sought to 1) evaluate an scFv based CD47-CAR T cell product with a lower affinity antigen-binding domain (B6H12) and 2) generate inducible CD47-CAR T cells for functional characterization. As CV1 has a high affinity for CD47 (K_D ~11pM), we developed a second CD47-CAR based on the B6H12 ScFv (K_D ~5.27nM). Similar to CV1-CAR T cells, B6H12-CAR T cells downregulate CD47 surface expression and eliminate non-transduced T cells post-transduction resulting in a ~100% B6H12-CAR positive population. We previously found robust expansion of CV1-CAR T cells preceded by an initial lag in expansion post transduction, however B6H12-CAR T cells had significantly impaired expansion that did not recover at any time post transduction. Despite limited expansion, B6H12-CAR T cells exert antitumor activity in vitro and, consistent with CV1-CAR T cells, lack antitumor activity in vivo likely due to macrophage mediated elimination. Given that multiple constitutively active CD47-CAR T cell products downregulate CD47 and fail to function in vivo, we generated CD47-CARs under control of a doxycycline inducible (Tet-On) promoter that constitutively express ZsGreen (ZsG) as a detection marker (indB6H12- and indCV1-CAR). In the absence of doxycycline, indB6H12- and indCV1-CAR CAR T cells have no impaired expansion. Upon treatment with doxycycline ind.B6H12-CAR T cells had high CAR expression, decreased surface CD47 expression, and are capable of killing CD47 positive targets in vitro. Interestingly, when indCV1-CAR T cells were treated with doxycycline, no CAR was detected on the cell surface despite expressing ZsG, which confirms successful transduction. This suggested the indCV1-CAR may be expressed, albeit sequestered within T cells by binding intracellular CD47. We therefore asked if indCV1-CARs are expressed in CD47 negative cells (OV10). Indeed, upon treatment with doxycycline the indCV1-CAR was expressed on the surface of OV10 cells. Overall, these results demonstrate the CAR binding domain influences CD47-CAR T cell functional characteristics in both constitutive and inducible systems. While studies are ongoing, we demonstrate that 1) similar to CV1-CAR T cells, B6H12-CAR T cells downregulate CD47 surface expression and lack antitumor activity in vivo and 2) CD47-CAR binding domain determines CAR surface expression upon activation in an inducible system. We speculate that CAR affinity or binding site on CD47 is responsible for differing CD47-CAR T cell functional phenotypes and studies are underway to elucidate mechanisms of these findings.

615 Dissecting Gene Networks in LV-Targeted Phagocytic Cell Populations

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Viral vectors are versatile and efficient tools for genetic engineering of eukaryotic cells. Among these, VSV-G-pseudotyped lentiviral vectors (LVs) have been extensively investigated for their capacity to stably transduce non-proliferating cells. Upon intravenous injection in non-human primates and mice, LVs efficiently transduce numerous cell populations in the liver and to a lesser extent in the spleen. Thus, LVs can be efficiently employed to drive transgene expression in well-defined hepatic or splenic cell populations. Albeit *in vivo* LV-transduced cell populations and the transcriptional networks active in said cell types have been described, an in-depth molecular characterization is only partially known. Understanding LV biodistribution and dissecting the molecular circuits that govern the genetic functions of LV-transduced cells, may be of key relevance to exploit systemically delivered LVs in distinct pathological conditions. We delivered GFP-encoding LVs to adult mice through tail vein injection and observed transgene expression by flow cytometric and immunofluorescence analyses. We identified several distinct splenic and hepatic cell populations that were transduced by LVs. The genetic programs of these cells in healthy tissue and in the presence of liver metastases were characterized by employing total and small RNA sequencing analyses. We identified key microRNAs (miRNAs) that may regulate important biological functions in liver resident macrophages, Kupffer cells. By measuring miRNA activity at single cell level, we found that miR-342-3p was downregulated in Kupffer cells in the presence of liver metastases, suggesting an anti-tumor function of this miRNA. Building on this observation, we enforced miR-342-3p expression in Kupffer cells in mice bearing liver metastases by employing systemically delivered LVs. We found delayed liver metastasis growth and immune activation, suggesting that restoring miR-342-3p expression level may reprogram Kupffer cell functions from protumoral to antitumoral. Moreover, we identified putative miRNA targets that may fine tune KC phenotype. Overall, our work provides insights on the populations targeted by systemic LV biodistribution and identifies active gene networks, which may be of interest for improving in vivo gene engineering approaches, e.g. by selecting promoters or miRNA target sequences to specifically drive gene expression in desired cell populations. Furthermore, we identified an antitumoral function of miR-342-3p in Kupffer cells in the presence of liver metastases.

616 A Scalable, iPS Cell Derived Lymphocyte and Myeloid Multi-Cell-Type Therapeutic Platform for Enhanced Tumor Cell Killing

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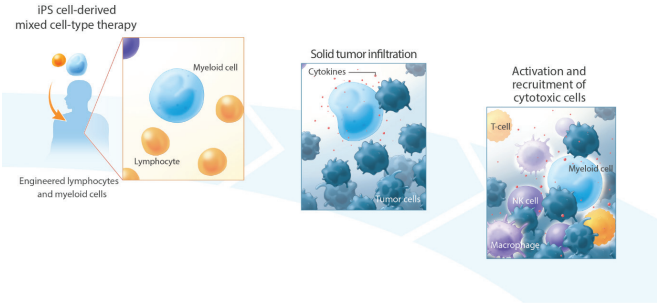
Induced pluripotent stem (iPS) cell therapies have the potential to treat a wide variety of devastating diseases. iPS cell-derived lymphocytes (e.g., T cells and NK cells) engineered to express targeting molecules such as chimeric antigen receptors (CARs) have shown clinical promise to treat hematological malignancies. More recently, iPS cell-derived myeloid cells are being developed to treat both hematological malignancies and solid tumors due to the ability of these cells to infiltrate and modulate the tumor microenvironment. Despite preliminary success, several challenges still remain, including poor infiltration of cytotoxic lymphocytes into solid tumors and insufficient cytotoxicity of myeloid cells. We hypothesized that a multi-cell-type therapy comprising both lymphocyte and myeloid cells may work synergistically, enhancing cytotoxicity and efficacy. We previously demonstrated the directed differentiation of mRNA reprogrammed iPS cells into functional cytotoxic lymphocytes and monocyte-derived M1 and M2 polarized macrophages. Here we describe a scalable bioreactor-based process for parallel differentiation of mRNA reprogrammed iPS cells into both CD14+ (>95% positive) macrophages and $CD56^{bright}/CD16^{dim}$ NK cells. This process yields 1x10^6 myeloid cells/mL and 3x10^5 lymphoid cells/mL, and is amenable to scaling to clinically relevant doses. In vitro, the lymphoid and myeloid cells showed synergistic tumor cell killing of SKOV3 ovarian cancer cells (combined: 15.6%; macrophage alone = 2.2% ($p<0.01$); NK alone = 7.5% ($p<0.05$); E:T = 5:1). The combined cells showed increased expression of TNF-α, and demonstrated enhanced clustering and tumor cell engagement. To further improve the macrophages' ability to target and infiltrate solid tumors, we transfected macrophages with mRNA encoding a humanized ROR1-CAR protein. mRNA transfection increased cytotoxicity towards SKOV3 cells by 6-fold. Here we describe a scalable platform for generating iPS cell-derived multi-cell-type therapies comprising both lymphoid and myeloid cells. We demonstrate that, much like the natural cellular immune response, these cells act synergistically to kill tumor cells in vitro. By more closely mimicking natural cellular immunity, multi-cell-type cell therapies represent a new class of cell therapies that may play an important role in the development of new medicines to treat cancer.



617 RNA-Based Therapy for Solitary Fibrous Tumors

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Solitary fibrous tumors (SFT) are rare, soft tissue tumors of mesenchymal origin that account for less than 2% of all soft tissue sarcomas. In the latest WHO classification published in April 2020, SFTs are subdivided into three categories: benign (locally aggressive), NOS (rarely metastasizing), and malignant. These tumors traditionally present unique diagnostic challenges because of the common gross and histologic features they express. A breakthrough in their characterization occurred in 2013, when all SFTs were found to exhibit a genetic fusion between NAB2 and STAT6 on chromosome 12. Despite advances in the classification and understanding of the molecular mechanisms of SFTs, no systemic therapeutic options are yet available. CRISPR (Clustered Regularly Interspaced Palindromic Repeats) technology has revolutionized the field of genetic engineering. Various CRISPR-mediated DNA editing technologies, such as the type II CRISPR/spCas9 and type V CRISPR-Cas12 (Cpf1) systems, have been extensively studied and already administered to patients during clinical trials. In parallel, efforts were also made to discover and establish diverse RNA-manipulating Cas effectors. More recently, the Type VI CRISPR/RfxCas13d (CasRx) system, which is derived from *Ruminococcus flavefaciens* XPD3002 and possesses dual RNase activities, was found to efficiently process target RNAs in mammalian cells with fewer non-specific targeting effects than other RNA-editing technologies, such as shRNAs (short hairpin RNA). All NAB2-STAT6 fusion types in SFTs create unique fusion RNA transcripts which are distinct from wild type NAB2 or STAT6 transcripts. In this study, we investigate CasRx-based RNA targeting technologies for the suppression of NAB2-STAT6 fusion transcript expression levels. We hypothesize this will exert anti-tumor benefits for SFTs with fewer off-target effects. Using CRISPR-mediated knock-in technology, we have successfully established stable SFT cell models harboring the NAB2-STAT6 fusion types. In addition, we have established primary cell models from patient tumor samples. We have developed CasRx-based constructs packaged into AAV viral vectors that exhibit dose-dependent suppression of specific NAB2-STAT6 fusion transcripts. Our work provides evidence of the efficiency of RNA-based therapy *in vitro* and aims to accelerate solitary fibrous tumor research.

618 Gene Editing and Immunotherapeutic Targeting of ADGRE2/EMR2 to Enable Combinatorial Targeting in AML

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Introduction: Acute myeloid leukemia (AML) is a heterogeneous disease characterized by abnormal clonal expansion. Targeted

immunotherapy of AML has been limited due to lack of true tumor-specific antigens. We developed an approach for increasing the therapeutic index and safety of targeted therapies by genetically ablating biologically dispensable target antigens from hematopoietic stem cell grafts (HCT), thereby restricting these antigens to AML cells, and enabling subsequent immunotherapy without risk of on-target off-tumor toxicities. Simultaneous targeting of multiple AML antigens may be required to help avoid potential antigen escape and address the issue of tumor heterogeneity, while ablation of the targets in the HCT by multiplex genome editing is necessary to protect the transplanted healthy graft. **Methods/Results:** To elucidate surface expression heterogeneity and prioritize antigens for combinatorial targeting, we performed quantitative flow cytometry surface expression profiling on validated AML targets, CD33, CLL1, and CD123, as well as a promising target ADGRE2/EMR2. Cross-sectional and longitudinal assessments of patient bone marrow (BM) was performed in >25 diagnosis (Dx) and >25 relapse samples to define the antigen positivity of blasts and leukemic stem cells (LSCs; CD34+CD38-), as well as antigen density (molecules per cell). The results demonstrate that these antigens are expressed on blasts and LSCs at targetable antigen densities (>1000 molecules per cell), but there is inter- and intra-patient antigen heterogeneity on both blasts and LSCs, suggesting that combinatorial targeting is necessary to fully eradicate leukemic cells in more patients. Healthy BM and peripheral blood flow cytometric analysis shows that these antigens are expressed on hematopoietic stem and progenitor cells (HSPCs) and myeloid lineages, suggesting that ablative editing may be necessary to protect healthy hematopoietic lineages from subsequent immunotherapeutic targeting. Here, we describe the ablative genome editing approach paired with a novel immunotherapeutic targeting for EMR2. Human genetic evidence of *ADGRE2* null individuals with no deleterious phenotype (https://gnomad.broadinstitute.org/) provides compelling evidence of biological dispensability. We utilized CRISPR/Cas9 and base editing technologies to genetically ablate EMR2 in HSPCs, achieving >80% editing with corresponding surface protein loss. Deletion of EMR2 did not impact *in vitro* myeloid differentiation or cytokine production as compared to unedited control cells. To develop EMR2-directed CAR-T cells, we performed a phage display screen to identify novel EMR2-specific human scFv and VH binders. CAR constructs encoding these binders were transduced into primary T cells and tested in cytotoxicity assays against the EMR2$^+$ MOLM13 AML cell line and EMR2-lacking cells. We observed EMR2-specific activation, cytokine secretion and potent killing. **Conclusion**: Our results identify AML antigens for combinatorial targeting and demonstrate feasibility of a novel EMR2-directed AML therapy paired with EMR2 deleted HCT. Moreover, our data provide a rationale for future combination of EMR2 with other AML antigens in a multiplex-editing approach paired with multi-antigen specific immunotherapy to enable next-generation AML treatments.

619 Screening and Identification of Optimal Costimulatory Domains for iPSC Derived CAR-NK Cells Targeting GPC3 Expressing Hepatocellular Carcinoma

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Introduction: Given the distinct expression profiles of costimulatory receptors in NK and T cells we set out to identify optimal costimulatory domains for the design of our iPSC derived CAR (chimeric antigen receptor) NK cell platform for targeting GPC3 expressing Hepatocellular Carcinoma (HCC). To this end we designed both 2nd and 3rd generation CAR constructs containing costimulatory domains that are specific for NK cells or shared with T cells and evaluated their NK cell cytotoxic function against GPC3 expressing HCC tumors. **Methods:** The following 2nd and 3rd generation CAR constructs containing GPC3 scFV binder (YP7 mAb) and CD28 hinge and transmembrane domains were expressed with the following costimulatory domains: CD28+CD3 zeta (2ndgeneration), 41BB+CD3 zeta (2nd generation), 2B4+CD3 zeta (2nd generation), CD28+41BB+CD3zeta (3rdgeneration). The CAR constructs were expressed in either EF-1a promoter containing vectors for electroporation into NK-92 cells or in gene of interest pAAV6 shuttle vectors for AAV6 production for targeted gene locus knock in (Beta 2 microglobulin locus) with locus specific sgRNA and Cas9 or into retrovirus shuttle vectors for production of retroviral particles containing BaeV envelope proteins for transduction of PBNK cells. Transduced PBNK cells were expanded in K562 feeder cells expressing 41BB ligand and membrane bound IL-21. NK-92 and PBNK cells expressing CARs were phenotyped for CAR expression using soluble GPC3 FITC and tested for cytotoxicity against Hep3B tumors by flow cytometry or in tumor spheroids killing assays using special U-bottom adhesive plates and in serial tumor killing assays using the Incucyte™ Live Cell Analysis System. **Results:** Given the targeted gene loci specific (B2M gene locus) non-disruptive gene knock in approach considered for the iPSC-CAR NK cell platform, we initially focused on AAV6 transduced PBNK cells where the 2nd and 3rdgeneration CARs were knocked in to the B2M locus. Both PBNK donors showed 80-90% of the cells expressing GPC3 CAR and non-disruptive B2M expression suggesting this was a viable approach to drive CAR expression at this locus. In both Hep3B tumor spheroid killing and Hep3B tumor serial killing assays as expected all PBNKs expressing CARs showed significantly higher Hep3B tumor killing compared to control non-transduced PBNKs. Second generation 2B4 CARs showed the highest cytotoxicity compared to all other 2nd generation and 3rd generation CARs with the second generation CD28 CAR showing the least anti-tumor killing activity in both tumor spheroid and serial killing assays. Although CAR expression in retroviral transduced PBNKs was lower (45-50%) in all 2nd and 3rdgeneration CARs, the cytotoxic activity against Hep3B tumors was comparable except for the lower cytotoxicity observed with the CD28 CAR. A similar trend was observed in NK92-CD16 cells expressing 2nd and 3rd generation CARs against Hep3B tumors. These results suggest that NK cell expressed costimulatory domain CAR's show higher cytotoxic activity compared to the T cell specific CD28 costimulatory domain CARs expressed in NK cells. **Conclusion:** The above data enabled the selection of optimal CAR constructs candidates for the derivation of iPSC derived CAR-NK cells against HCC tumors.

620 Development of a Ferret Model of Idiopathic Pulmonary Fibrosis by Bleomycin-Induced Lung Injury

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Idiopathic Pulmonary fibrosis (IPF) is a progressive and irreversible disease characterized by lung scarring and remodeling with excessive extracellular matrix (ECM) deposition and destruction of lung architecture that ultimately leads to pulmonary function decline and respiratory failure. IPF is the most common and severe form of fibrotic disease in lung with unknown etiology. To date, IPF remains an incurable disease with limited therapeutic options, novel therapeutic targets and strategies are therefore unmet needs for treatment of this lifelong debilitating disease. Gene and cell therapies offer promising avenues to treat a range of fibrotic diseases including IPF. However, the lack of appropriate animal models that are able to recapitulate the human disease feature significantly hinders *the* development of these novel treatments for IPF. While the existing bleomycin (BLM)-induced mouse and rat PF models *has* provided important insights into the pathophysiology of IPF, they fail to reproduce the progressive and irreversible features in IPF patients. Domestic ferrets are recognized as excellent models for medical research of respiratory diseases such as influenza infection, chronic obstructive pulmonary disease (COPD), obliterative bronchiolitis (OB), emphysema caused by alpha-1 antitrypsin deficiency and cystic fibrosis (CF), owing to similarities of airway anatomy, lung cell biology and physiology between ferrets and humans. However, the IPF phenotype in BLM-injured lungs of ferrets has yet been systematically characterized. Here, we evaluated changes in histopathology, pulmonary function, and biomarkers of IPF in ferrets injured by lung challenge with 3 doses of BLM (2.5U. mL-1·Kg-1, 1 week interval). Histopathological analysis showed higher Ashcroft scale scores of pulmonary fibrosis with predominant deposition of collagen and ECM in lungs of ferrets challenged with BLM, compared to healthy ferrets. Pulmonary function test (PFT) demonstrated that the BLM exposures led to the decrease of pulmonary compliance, a typical phenotype of IPF as accessed using the FlexiVent at 4 and 8 weeks post the first BLM challenge. Notably, more severe IPF phenotype was observed in male and older animals compared to female and younger compartments, respectively, as determined by the degree of pathological change and pulmonary function decline. Molecular studies further demonstrated an increase in ECM deposition, epithelial-mesenchymal transition, fibrotic biomarkers of Krebs von den Lungen 6, MUCIN 5B (MUC5B), surfactant protein C (SFTPC) and cytokeratin 8 in lung tissues of BLM-challenged ferrets. In addition, increased abundance of Krebs von den Lungen 6, S100 calcium-binding protein A12, SFTPA, SFTPD, MUC5AC, MUC5B and matrix metalloproteinases were also observed in plasma of IPF ferrets. Together with results of histopathology and PFT, this study provides evidence that the BLM-induced IPF ferrets can reproduce clinical, pathological and molecular features, and disease course observed in IPF patients, indicating that IPF ferrets are a reliable model for understanding mechanisms of IPF pathogenesis and testing therapeutic strategies and agents, including gene and cell therapy for IPF treatments.

621 TNS3 Deficiency Upregulates TGF-β/Smad Signaling and Contributes to Fibrogenic Progression

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Idiopathic pulmonary fibrosis (IPF) is a progressive and smoking-associated lung disease, with a poor prognosis and limited therapeutic options. Targeting activated fibroblasts, mainly myofibroblast differentiation, has emerged as a therapeutic strategy in IPF management; however, the viable signaling molecules associated with fibroblast activation are yet to be identified. To uncover the regulatory molecules that drive gene expression representative of IPF features in the lung, we analyzed the single-cell RNA sequencing data and noticed dysregulation of integrin-mediated focal adhesions in IPF lung fibroblast populations such as the myofibroblast subpopulation, a key player in fibrogenesis. Of these focal adhesion molecules, a prominent downregulation of Tensin-3 (TNS3), a tensin family member, was noted in IPF lung fibroblasts, whereas no significant change on TNS3 expression was seen in other lung cell types. Analysis of the Comparative Toxicogenomics Database revealed a strong interaction of TNS3 with byproducts of cigarette combustion. Immunoblotting analysis confirmed that normal lung fibroblasts display higher expression of TNS3 compared to that in lung fibroblasts derived from IPF patients. Exposure to either cigarette smoke extracts or a profibrotic factor, TGF-β, downregulates TNS3 expression in lung fibroblasts. This downregulation was also noted in the fibroblastic foci of lung tissue sections from IPF patients by using immunohistochemistry staining. Genetic knockdown of TNS3 studies demonstrated that TNS3 loss activates a critical member of the TGF-β pathway, Smad2, and increases expression of the myofibroblast marker α-SMA. In a bleomycin-induced pulmonary fibrosis model, TNS3-knockout mice manifested severe loss of body weight, a worse survival rate, and extensive lung structural changes and collagen deposition than wild-type mice did. Taken together, our results not only provide a novel understanding of how TNS3 loss mediates fibroblast cell activities but also suggest a potential biomarker for IPF.

622 Mending Broken Ends: AAV Gene Therapy Targeting Telomeric Ends Restores Cardiac Function in Ang2, TAC, and CKD Heart Failure Mouse Models

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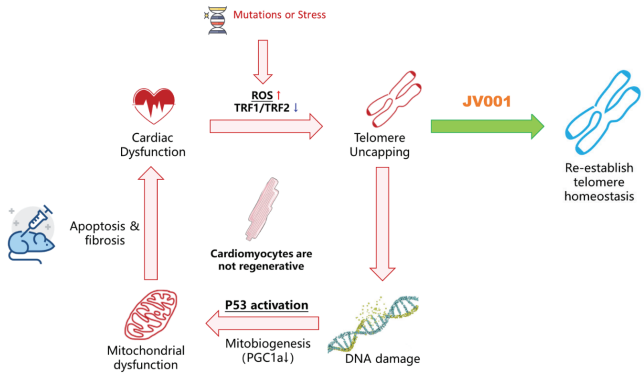
Cardiovascular disease and heart failure is among the top causes of morbidity and mortality worldwide with a critical need for innovative therapies to reverse the course of ventricular dysfunction. In previous studies, we established that genetic cardiomyopathic cardiomyocytes exhibit accelerate telomere shortening which leads to DNA damage p53 activation and inhibition of mitochondrial biogenesis. Here we present results from three general heart failure mouse models: TAC surgery to model Hypertrophy Cardiomyopathy/HFpEF, Ang2 infusion to mimic Dilated Cardiomyopathy/HFrEF, and 5/6 nephrectomy to mimic chronic kidney disease induced heart failure. Dose response experiments followed by biodistribution and histological examination show good safety profile. Using AAV9 and the cTnT (cardiac troponin T) promoter to express a telomeric capping protein, JV001, heart failure animals (LVEF drops from baseline ~80% to ~50%) were treated with a single *i.v.* dose. An increase of ~20% LVEF were observed in all three heart failure mouse models by 7-10 days post administration and efficacy remained stable at 1-year sacrificial endpoint. Histologically, JV001 treatment prevented further telomere attrition and myocardial loss/cardiac fibrosis. Using live cell labeling and electron microscopy, we confirm that JV001 treatment successfully relieved mitochondrial biogenesis blockage. JV001 treatment also arrested telomere attrition and improved contractile function in Ang2 treated hiPSC-CM model.



623 Concurrent Knockout of RYR2 and Activation of RYR1 or RYR3 in Cardiomyocytes: A Potential Therapy to Rescue Catecholaminergic Polymorphic Ventricular Tachycardia?

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Cardiac excitation-contraction coupling describes the process from the electrical excitation of cardiomyocytes to their contraction. It relies on the concerted action of Cav1.2 channels in the T-tubule system and ryanodine receptors 2 (RYR2), the predominant calcium release channels in the sarcoplasmic reticulum. Disruption or dysfunction of RYR2 results in impairment of cardiomyocyte maturation or occurrence of ventricular arrhythmias, such as Ca^{2+}-release deficiency syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT1). In mammals, the genome encodes for two additional RYR isoforms which are structurally and functionally highly related but predominantly expressed in the skeletal muscle (RYR1) and the brain (RYR3). Gene supplementation therapy for patients carrying mutations in RYR2 is hardly possible as the coding sequence of this gene (≥16.5 kb) exceeds the packaging capacity of adeno-associated viral (AAV) vectors (4.7 kb). Moreover, CPVT1 is caused by various autosomal dominant gain-of-function mutations in *RYR2*. This suggests that depletion of this gene, along with expression of another gene that can compensate for the lack of RYR2 function (e.g., RYR1 or RYR3), may be suitable to achieve therapeutic benefit. To provide a proof-of-principle for concurrent knockout and activation of different genes in murine cardiomyocytes, we used the Cas9-VPR module in combination with single guide RNAs (sgRNAs) with different spacer lengths, i.e., shortened spacers (< 15 nucleotides) for transcriptional activation and regular spacers (20 nucleotides) for knockout. To deliver Cas9-VPR (5.8 kb) and the sgRNAs driven by a heart-specific cTnT-promoter to the heart, we intraperitoneally injected mice with dual mRNA trans-splicing AAV9 vectors. To distinguish the respective effects of *Ryr2* knockout and *Ryr1* activation on cardiac phenotype, we injected dual AAVs expressing different combinations of sgRNAs in three cohorts: Cohort #1 was expressing one sgRNA for *Ryr2* knockout alone, cohort #2 one sgRNA for *Ryr2* knockout and two sgRNAs for *Ryr1* activation and cohort #3 two sgRNAs for *Ryr1* activation. Analysis of isolated cardiac tissue using qRT-PCR and Western blot experiments confirmed highly efficient *Ryr2* knockout and/or *Ryr1* activation. Isolated hearts of cohort #1 and cohort #2 showed cardiac remodeling with hypotrophic ventricles and significantly enlarged, dilated atria. In the ECG, the lack of RYR2 was manifested in widened P waves and a reduction in the amplitude of QRS complexes compared to control. At the cellular level, morphological changes of cardiomyocytes with disruption of t-tubules were observed. No obvious cardiac abnormalities were detected in cohort #3, suggesting

that RYR1 expression has no adverse effects on cardiac function. Taken together, this study reinforces the critical role of RYR2 in SR homeostasis and in maintaining T-tubule integrity in mature cardiomyocytes. Cardiac defects caused by depletion of RYR2 could not be rescued by transcriptional activation of *Ryr1*. Further experiments are underway to verify whether RYR3 can functionally replace RYR2 in cardiac tissue.

624 Effects of AAV-*TAFAZZIN* in Barth Syndrome Patient-Derived iPSC Cardiomyocytes and Myoblasts

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Barth syndrome (BTHS) is a rare, X-linked, mitochondrial disease that is caused by mutations in the gene TAFAZZIN. The tafazzin protein is an acyltransferase that remodels the inner mitochondrial membrane protein monolysocardiolipin to mature cardiolipin. BTHS causes severe cardioskeletal myopathy, impaired cardiac and skeletal muscle bioenergetics, and neutropenia. Our prior preclinical studies demonstrated the ability of adeno-associated virus (AAV)-mediated gene therapy to improve heart and skeletal muscle function in a mouse model of BTHS using a wide variety of functional and quantitative proteomic analyses. In addition, we have shown that delivery of AAV-TAFAZZIN to BTHS patient-derived fibroblasts restored mtDNA copy numbers, mitochondrial structure, and function. To further develop our gene therapy approach, we have differentiated healthy and BTHS patient-derived iPSCs into mature cardiomyocytes (iPSC-CMs) and myoblasts (iPSC-Mbs) to test the effects of AAV-mediated gene delivery on disease phenotypes. Our protocol combines both medium-based and substrate stiffness based maturation techniques and yielded more metabolically mature iPSC-CMs based upon electron transport chain complex activities and oxygen consumption assays. Administration of 50,000 vg/cell of AAV-TAFAZZIN for seven days resulted in high level (500-1,500 fold increase) TAFAZZIN expression and significant improvements in spare capacity and other oxygen consumption based assessments in treated BTHS cells as compared to controls. In order to characterize the disease mechanisms of BTHS more fully, a tandem mass tagging (TMT) based proteomics analysis study was performed to compare protein expression profiles between mature human healthy and BTHS-patient derived iPSC-CMs. These data are currently being processed to identify underlying mechanisms of cardiomyocyte dysfunction that represent novel therapeutic targets that are relevant to other cardiomyopathies. A BTHS proteomics study we previously performed to evaluate the effect of AAV-mediated gene therapies on the global proteome of BTHS mouse hearts as compared to healthy controls revealed significantly decreased levels of several proteins that may represent unifying therapeutic targets for heart disease. We have generated an AAV vector to test the effects of overexpression of one of these as compared with two different isoforms of TAFAZZIN in BTHS patient-derived iPSC-CMs and iPSC-Mbs. Ongoing studies include completion of in vitro proteomics assessments, performing a panel of comparative mitochondrial functional assays, and assessing the effects of these treatments on mitochondrial structure and dynamics. Completion of these studies will support further translation of AAV-TAFAZZIN gene therapy towards human clinical trials for individuals with BTHS.

625 Barcoded AAV Libraries Identify Serotypes Capable of Transducing Fibroblasts in Mouse Lungs

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Idiopathic pulmonary fibrosis (IPF) is a complex polygenic disease characterized by the stiffening of the lung and pulmonary fibrosis that is modulated by several cell-types in the lung such as fibroblasts, epithelial alveolar type I (AT1), and type II cells (AT2). While the vast work on the monogenetic disease, cystic fibrosis, has identified AAV serotypes with tropism for epithelial cells, to date there are no examples of AAVs that enable lung fibroblast transduction. In order to enable gene delivery into lung fibroblasts, we have developed a platform that couples single-cell sequencing with barcode expression to both screen AAV serotypes for lung fibroblast transduction and optimize regulatory elements that confer high expression of the transgene payload in this cell type. We performed oropharyngeal injections of a library of 16 barcoded serotypes, at a dose of 1E10 vg/serotype, into several bleomycin-induced IPF model mice as well as non-diseased mice (n = 3 mice for each model). Our resulting screen correctly identified AAV6 as a serotype that effectively transduces AT1 and AT2 cells. Additionally, we identified several serotypes capable of transducing fibroblasts with high efficiency. These serotypes were then individually validated in mice through histology and FACS with a dose of 2E10 vg ($n \geq 2$ for each serotype) giving transduction percentages from 1% to 10% of all fibroblasts transduced. Increasing the dose to 7E11 vg obtained transduction of fibroblasts at 31%. Interestingly, we saw differences in transduction efficiency across subsets of celltypes; the activated myofibroblasts in the IPF mouse model were more likely to be transduced than the lipofibroblasts of the non-diseased mouse model. Thus, by validating serotypes in a single-cell manner in a relevant disease model, our platform can identify differences in AAV tropism to different cell states. The same barcoding and single-cell screening approach was next used to optimize regulatory elements for a gene of interest for the highest relative number of transduced cells and level of expression across different cell-types within the mouse lung. A library consisting of 47 different combinations of promoters, enhances, introns, and polyA signals was delivered via the oropharyngeal route to IPF and healthy mice (n = 10). Fibroblasts were isolated and by sequencing RNA transcripts, we identified a promoter and intron that resulted in transcripts and expression detectable in 2x as many cells as a ubiquitous CMV promoter. Combined, our results have identified a serotype and promoter that enables high expression and targeted gene delivery to mouse lung fibroblasts.

626 Site-Specific Editing Methods to Reverse Severe Combined Immunodeficiency (SCID) in Athabascan-Speaking Native Populations

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Severe combined immunodeficiency of Athabascan-speaking Natives (SCID-A) arises from a single C→A nucleotide substitution in exon 8 of the DNA cross-link repair 1C (*DCLRE1C*) gene, resulting in a premature stop codon (pmSTOP) and a non-functional Artemis protein. The absence of Artemis results in an impaired canonical non-homologous end joining NHEJ (c-NHEJ) DNA damage repair and arrested V(D)J recombination, which leads to hypersensitivity to DNA-damaging agents, a $T^-B^-NK^+$ immunophenotype, and susceptibility to severe infections. *Ex vivo* lentiviral therapy of Artemis in SCID-A hematopoietic stem cells (HSCs) has been clinically tested, but shows suboptimal expression and carries the risk of insertional mutagenesis. Here, we demonstrate an alternative, potentially superior approach for site-directed genetic repair of the Artemis-encoding sequence *in situ*, correcting the pmSTOP founder mutation using adenine base editor (ABE). The ABE system can create a site-specific, irreversible DNA edit to convert the pathogenic pmSTOP codon into a conserved tryptophan (TAA→TGG), thereby restoring Artemis activity. We have validated this substitution *in vitro* through assays measuring enzymatic hairpin-opening activity and cell proliferation following ionizing radiation in model K562 lines encoding the SCID-A mutation or the tryptophan conversion **(Fig 1A-C)**. Using the ABE8e-NG variant, we achieved >40% pmSTOP→tryptophan sequence conversion in SCID-A K562s, with the restoration of Artemis validated by Western blot and enzymatic activity assays underway. Furthermore, to facilitate pre-clinical evaluation of *in vivo* correction, we generated a novel murine model of SCID-A by electroporating mouse embryos with Cas9-ribonucleoprotein and transducing them with a recombinant adeno-associated virus (rAAV) donor molecule **(Fig 2A)**. In this model, exon 8 of the murine *Dclre1c* locus was replaced with the human wild-type or mutant *DCLRE1C* human sequence, the latter which recapitulates the SCID-A $T^-B^-NK^+$ phenotype and the absence of Artemis **(Fig 2B-C)**. To test ABE-mediated correction in the mouse model *ex vivo*, we targeted the mutation in lineage-depleted SCID-A hematopoietic stem and progenitor cells (HSPCs) and transplanted them into lethally-irradiated SCID-A recipients. We anticipate that transplanted ABE8e-NG-edited progenitor cells will result in the appearance of significant levels of T or B cells in the peripheral blood, and further analyses will show the restoration of Artemis protein and its endonucleolytic activity within the outgrown cells. Our work demonstrates this site-specific approach's broader potential for treating other primary immunodeficiencies and genetic diseases using gene-corrected autologous transplants and *in vivo* delivery.

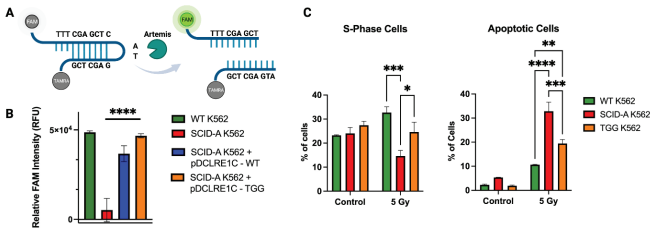

Figure 1. Validation of restoration of Artemis protein activity assays.
A, Artemis enzymatic hairpin-opening activity using a synthetic hairpin with FAM fluorophore at 5' and TAMRA quencher at 3' cleaved by Artemis. **B,** Enzymatic activity of Artemis in WT K562, SCID-A K562, SCID-A K562s encoding WT plasmid and SCID-A K562s encoding conservative tryptophan (TGG) plasmid measured after 6 hours of exposure to synthetic hairpin. **C,** Percentage of S-phase (left) and apoptotic (right) K562 model cells lines after exposure to 5 Gy of radiation.

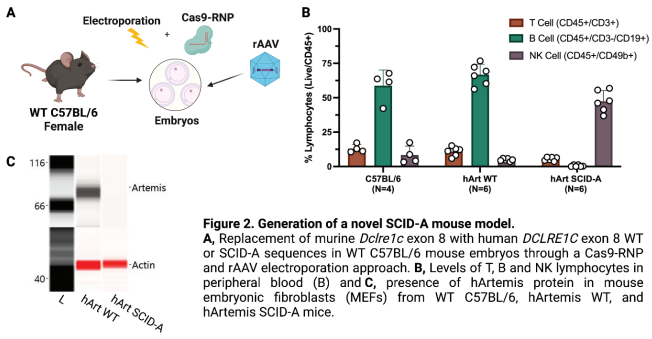

Figure 2. Generation of a novel SCID-A mouse model.
A, Replacement of murine *Dclre1c* exon 8 with human *DCLRE1C* exon 8 WT or SCID-A sequences in WT C57BL/6 mouse embryos through a Cas9-RNP and rAAV electroporation approach. **B,** Levels of T, B and NK lymphocytes in peripheral blood (B) and **C,** presence of hArtemis protein in mouse embryonic fibroblasts (MEFs) from WT C57BL/6, hArtemis WT, and hArtemis SCID-A mice.

627 Long Term Expression of Anti-Sickling Transgenic Beta-Globin after Non-Myeloablative Conditioning Lentiviral Gene Therapy in a Sickle Cell Disease Murine Model

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Sickle cell disease (SCD) results from a sequence defect in the adult hemoglobin (HbA) β-globin chain. This disease causes acute and chronic multiorgan failure, and reduced lifespan despite current advances in medical management. To improve these outcomes, gene therapy is being studied in the preclinical and early clinical setting. We have engineered a novel lentiviral vector based on the Lenti/βAS3-FB construct. The βAS3-globin transgene expressed from this vector contains the mutations G16D, E22A and T87Q that increase affinity for healthy β-globin and inhibit axial and lateral contacts with sickle β-globin, thereby conferring anti-sickling properties. We have successfully used our vector in a gene therapy model in the Townes mouse model for SCD. Recipients underwent a non-myeloablative conditioning regimen of 6Gy whole body irradiation and each received 1 million donor cells via retro-orbital injection. We optimized the survival of the mice by implementing a transfusion program following cell injection. Furthermore, we successfully detected lentiviral vector-encoded βAS3-globin in peripheral blood after gene therapy. We followed these mice over 30 weeks post-BMT and report stable peripheral blood vector copy number as well as persistent βAS3-globin measured by mass-spectrometry (see our corresponding poster). We analyzed several parameters in these mice including some for the first time in a Townes mouse gene therapy setting. We assessed

complete blood counts (CBC), *in vitro* sickling of peripheral blood red blood cells, urine osmolality and urine albumin/creatinine ratio. We also completed non-invasive echocardiography, whole body non-invasive plethysmography, von Frey behavioral assay, and a pathologic evaluation by hematoxylin and eosin and Prussian blue staining in transplanted mice. Our findings suggest several readouts that will be useful in evaluating gene therapy approaches in the Townes mouse model of SCD.

628 HDAd6/35++ - A New Helper-Dependent Adenovirus Vector Platform for *In Vivo* Transduction of Hematopoietic Stem Cells

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In previous studies we achieved safe and efficient *in vivo* hematopoietic stem cell transduction in mobilized mice and macaques with intravenously injected helper-dependent adenovirus HDAd5/35++ vectors. These vectors are derivatives of serotype Ad5 containing CD46-affinity enhanced Ad35 fiber knob domains. Considering the impact of anti-Ad5/HDAd5/35++ neutralizing serum antibodies present in the human population, we generated HSC-retargeted HDAd6/35++ vectors derived from serotype 6. We found lower prevalence and titers of serum anti-HDAd6/35++ in human samples compared to HDAd5/35++, potentially allowing for efficient *in vivo* HSC transduction in a greater fraction of human patients. Intravenous injection of an integrating HDAd6/35++-GFP vector into mobilized mice resulted in efficient HSC transduction with subsequent stable, high-level GFP marking in peripheral blood, bone marrow, and spleen cells as well as in tissue macrophages derived from *in vivo* transduced HSCs. In contrast to HDAd5/35++, undesired transduction of hepatocytes was minimal with HDAd6/35++. Furthermore, HDAd6/35++ allowed for efficient *in vivo* HSC transduction in Ad5-pre-immune mice. These features together with the straightforward production of HDAd6/35++ vectors at high purity and yield, make this new HDAd vector platform attractive for clinical translation of the *in vivo* approach.

629 Safety and Efficacy of Liver-Directed AAV Gene Therapy in Privately Owned Dogs with Severe Hemophilia A

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Hemophilia A (HA) is a monogenic bleeding disorder caused by low circulating coagulation factor VIII (FVIII) which results in spontaneous hemorrhages. In humans, the standard of care of prophylactic FVIII infusions has recently been challenged by non-factor therapies; however, both carry the burden of routine administration. Liver-directed adeno-associated virus (AAV) gene therapy is a potential one-time treatment that can significantly ameliorate the disease phenotype. However, questions remain about possible AAV oncogenic risk and longevity of transgene expression after gene transfer. HA dogs housed in research colonies have been used as preclinical models in therapeutic studies due to the similarities of their disease phenotype and predictability of dose response. Studies in privately owned HA dogs, in addition to providing quality of life data, may provide a better real-world estimate of AAV gene therapy safety and efficacy in clinical practice. Here, we present long term data on AAV gene therapy in privately owned HA dogs. 11 privately owned dogs with severe HA were treated with AAV8 vectors to express canine (c) FVIII. Three vector constructs were employed, and doses ranged from $5.4x10^{12}$ to $2.5x10^{13}$ vg/kg. In all recipients with available data (n = 10), cFVIII activity increased to >1% normal, with a median cFVIII activity of 3.2% (range 1.1 - 13.8%) after 4.1 years (range 2.6 - 8.9 years). These FVIII transgene levels resulted in a 93% reduction in the annualized bleeding rate. No new anti-cFVIII antibodies developed after gene therapy. However, a pre-existing anti-cFVIII antibody resolved in one recipient (PC3) after gene therapy. 3 dogs have died during follow up; 2 dogs (PC1 and PC3) with cFVIII <3% died at 8.8- and 2-years post treatment due to bleeding events. The last dog, PC9, was euthanized 3 years after gene therapy due to pervasive multicentric lymphoma. PC9's B-cell lymphoma showed high levels of cancer infiltration in the spleen and lymph nodes. Samples of PC9's liver, spleen, and lymph nodes were analyzed for presence of the human alpha1-antitrypsin promoter present in the AAV vector. Liver samples were found to have a vector copy number (VCN) of .05, splenic samples had a VCN of .02, and lymph node samples had a VCN of .0006. These VCNs are consistent with the normal biodistribution of AAV8 in a dog and well below the expected VCN if AAV integration had driven oncogenesis. Further AAV integration analysis found no evidence of a substantially expanded clone harboring vector integrations. In conclusion, AAV-cFVIII gene therapy is effective at reducing bleeding events in privately owned dogs. AAV gene therapy eradicated

a pre-existing anti-cFVIII immune response in one dog. The only serious adverse event was the development of cancer in one dog, which was rigorously determined to be unrelated to the AAV gene therapy. Terminal bleeding events occurred in dogs with moderate cFVIII activity, highlighting the need for levels >5%. These results support the safety and efficacy of AAV gene therapy for HA.

630 Alpha Globin Lentiviral Vectors for Hematopoietic Stem Cell Gene Therapy of Alpha-Thalassemia

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α-thalassemia is an inherited blood disorder caused by mutations in α-globin genes (HBA1 and HBA2) resulting in the reduction of α-globin chains, the subunit, along with β-globin chain, constituting adult hemoglobin ($\alpha_2\beta_2$). Severe α-thalassemia arises with α-globin expression levels of <30%, and α/β-globin ratio of <0.3, caused by defects in or absence of three or all four α-globin genes. Treatment for survival entails lifelong, biweekly blood transfusions with daily iron chelation therapy. While these therapies enable patients to live into mid- to late-adulthood, they continue to engender serious clinical manifestations. To address this clinical need, our laboratory has developed a stem cell gene therapy in which functional copies of α-globin gene integrate into the genome of patient's hematopoietic and progenitor stem cells (HSPCs) by lentiviral vectors (LVs) to normalize the globin chain imbalance and restore hemoglobin function. The design of the α-globin LVs (AGLVs) is based on *GLOBE* β-globin LV utilized in a clinical trial for β-thalassemia, which has achieved transfusion independence in patients with transfusion-dependent β-thalassemia major. To target excess infective erythropoiesis and hemoglobin restoration, we have constructed a series of short proviral length AGLVs for optimized titer production, HSPC infectivity, and gene expression. We constructed twelve AGLVs varying in gene and regulatory element compositions. Each AGLV was assessed for its raw titer yield, gene transfer efficiency, α-globin mRNA expression, and hemoglobin production in an α-globin knockout (KO) HUDEP human erythroid cell line. Successfully, **all AGLVs confer high raw titers ~ 1e7 TU/mL and express α-globin chain yielding adult hemoglobin**. We identified two optimal AGLVs for further characterization in human primary HSPCs: *Alpha2* for yielding highest raw titer and gene transfer efficiency and *LCR-Globe* for producing highest α-globin mRNA expression. Both *Alpha2* and *LCR-Globe* LVs harbor the HBA2 gene regulated by the β-globin promoter, but they differ by the length of their β-Locus Control Region (LCR) enhancer. To assess candidate AGLVs in healthy human HSPCs, AGLVs were tagged at the transcription level to enable identification and quantification of vector-derived α-globin mRNA proceeding HSPCs transduction and erythroid differentiation. Although *Alpha2* demonstrated optimum $CD34^+$ infectivity and gene transfer, ***LCR-Globe* expressed up to ~30% α-globin mRNA per total β-globin mRNA per vector copy number** (VCN), which is in the range of wildtype α-globin levels produced by one endogenous α-globin allele (~25% α-globin mRNA per total β-globin mRNA). We further examined *LCR-Globe* $CD34^+$ infectivity, erythropoiesis, and hemoglobin correction in α-thalassemia major patient HSPCs harboring lethal homozygous deletion of both HBA1 and HBA2. These studies demonstrated *a high CD34+ infectivity and gene transfer, without utilization of transduction enhancers, and obtained a correction of α/β-globin mRNA ratio of ~0.4 while retaining a low VCN of ~2.* Such results in patient cells are encouraging for disease amelioration in severe cases of α-thalassemia. Additional studies are underway, examining homologous candidate murine AGLVs in an α-KO murine erythroid cell line. They will then be evaluated in a α-thalassemia murine model for disease correction *in vivo.* Completion of these studies will enable the identification of a therapeutic AGLV candidate for gene therapy for α-thalassemia.

631 UM171 Promotes Gene Transfer, Reduces Apoptosis and DNA Damage and Increases Engraftment of Gene Modified Peripheral Blood Hematopoietic Stem Cells from Sickle Cells Disease Patients

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Sickle cell disease (SCD) is one of the most common genetic blood diseases. Lentivirus transduced autologous hematopoietic stem cell transplantation is an effective therapeutic strategy for SCD. Enhanced gene transfer efficiencies, reduced manufacturing stress and higher yields of engrafting transduced hematopoietic stem cells (HSCs) are ultimate goals for improving clinical protocols that use autologous gene therapies. Here, we aimed to improve the transduction efficiency, stem cell fitness and reconstitution of SCD CD34+ cells transduced with a lentiviral vector (LV) containing BCL11A shmiR currently in clinical trials (Esrick et al. NEJM, 2021), to minimize potential damage during ex vivo culture and reduce the risk of accumulating genome damage. UM171, an HSC self-renewal pyrimidoindole derivative agonist, has been shown to expand HSCs and enhance multilineage blood cell reconstitution in mice. We tested the potential of UM171 to expand CD34+ stem cells derived from SCD peripheral blood (PB) HSCs and examined its effects on transduction, stem cell immunophenotype, apoptosis, cell cycle, DNA damage and engraftment. Culture of SCD CD34+ HSC in HSC expansion conditions with UM171 increased the proportion of CD34+CD38- cells and CD34+CD38-CD45RA-CD90+ long term HSCs (LT-HSCs), and the absolute number of CD34+CD38- cells and CD34+CD38-CD45RA-CD90+ LT-HSCs were increased 2- and 4-fold, respectively. Treatment with UM171 in vitro also significantly enhanced the transduction efficiency of BCL11A shmiR containing lentiviral vector (LV-BCL11A) in SCD CD34+ cells from 0.7 VCN to 1.0 VCN ($N = 5$, $P < 0.001$), and significantly decreased apoptosis of stem cells, and reduced DNA damage determined by γH2AX+ assays (see Table). We assessed the engraftment capability of SCD CD34+ transduced in the presence or absence of UM171 in NBSGW mice. UM171 increased the human CD45 engraftment of mice

measured in PB at 8 weeks post-transplantation (N = 3, P = 0.05) and enhanced the engraftment of lentivirus transduced in SCD CD34+-derived CD45+ cells (N = 3, P <0.05) (see Table). To assess the effect of UM171 more rigorously on functionally defined HSCs, we performed a competitive transplantation assay using CD34+ cells transduced in the presence vs absence of UM171. Analysis of PB obtained from the mice at 8 weeks post-transplantation showed that cells transduced in the presence of UM171 consistently outcompeted those transduced under control conditions, 57.5% compared with 42.5% (N = 9, P < 0.001). In summary, a short-time exposure of SCD CD34+ PB cells to UM171 enhances the LV-mediated transduction efficiency of HSCs derived from SCD patients, reduces DNA damage and apoptosis during ex vivo manipulation and enhances engraftment of gene modified cells in xenograft models. Implementing these findings in clinical gene therapy protocols may improve the efficacy and sustainability of gene therapy and generate new opportunities in the field of gene editing.

Functional phenotype of SCD stem cells transduce with LV-BCL11A in presence of UM171				
	Apoptosis of stem cells (%)	DNA damage (%)	Transduction efficacy (%)	Engraftment (%)
In absence of UM171	22.5 ± 6.7	10.6 ± 1.1	13.6 ± 2.6	6.3 ± 1.3
In presence of UM171	14.3 ± 2.4	6.6 ± 1.5	19.9 ± 2.9	9.3 ± 1.4
P value	< 0.001	< 0.001	<0.05	0.0502

632 Treatment of Canine Hemophilia A via Intraosseous Delivery of a Platelet-Specific Factor VIII-Lentiviral Vector

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Hemophilia A (HemA) is a genetic disease resulting from a factor VIII (FVIII) deficiency. Traditional protein infusion to treat HemA is costly and requires repeated dosing. We demonstrated previously that intraosseous (IO) gene therapy via delivery of lentiviral vectors (LVs) into bone marrow targeting FVIII expression in platelets successfully treated HemA mice, including mice that had developed FVIII inhibitors. To facilitate clinical translation, we investigated the treatment of HemA dogs using this approach. A lentiviral vector incorporating a platelet-specific promoter Gp1bα and canine FVIII gene was injected into the tibia/iliac bones of 5 HemA dogs (4 in Cohort #1, 1 in Cohort #2). Prior to injection, the dogs were treated with an immune modulation regimen to minimize the immune response. Following the procedure, blood samples were taken at various timepoints. All dogs recovered well from the procedure and had blood chemistry values within normal ranges. Expression of cFVIII was examined in platelets and plasma isolated from LV-treated dogs by ELISA and aPTT assays. Canine FVIII can be detected in platelets with the highest expression at 5-10 mU/10^8 platelets around 1-2 months post-procedure and expression persisted for the experimental duration in all treated dogs. There was no canine FVIII expression detected in treated dog plasma. The improvement of blood coagulation was evaluated by whole blood clotting time (WBCT) and thromboelastography. WBCT was shortened in multiple time points shortly after IO gene therapy, indicating improved hemostasis. Furthermore, the IO gene therapy was well tolerated and did not produce any toxicity as evaluated by CBC and blood chemistry analysis**.** Encouragingly, the dogs experienced fewer bleeding events per year after gene therapy treatment compared with the baseline prior treatment, indicating partial correction of HemA phenotype. For the first dog in Cohort #2, we incorporated several improvements over our previous protocol. Specifically, we performed injections in both the tibia and the iliac bone. Additionally, perioperative hemostasis was performed using bypassing agents to avoid inhibitor formation. At 3 weeks, canine FVIII can already be detected in platelets by ELISA with stable levels over the 2 months of follow-up. In conclusion, we have established an IO-LV gene therapy protocol to treat HemA dogs successfully with persistent effects of treatment over 2-4 years. Our study demonstrated a potential strategy for safe and effective application of gene therapy *in vivo* for treating HemA patients.

633 Destruction of T Cell Synapse Formation is a Novel Strategy for Hypoimmunity

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Allogeneic immune cells derived from engineered human induced pluripotent stem cells are becoming promising cell therapies for cancer. However, host-versus-graft rejection limits their durability in patients, particularly when the treatment involves multiple doses. The rejection mainly comes from host T cells. Knocking out of MHC-I/II has been proven to be effective for avoiding T cell attack while making the edited cells susceptible to host NK killing, due to the miss-self signal. Although numerous efforts have been applied to further engineer MHC-I/II KO with different NK inhibitory ligands, the high heterogeneity of NK cells renders it challenging to suppress all patient NK cells by expressing a few NK inhibitory factors. Alternatively, inhibiting host T cells without knocking out MHC-I could be a potential strategy for generating hypoimmunogenic cell products.In this study, we tested this alternative strategy by knocking out T cell second signaling molecules from T cell synapse for escaping host T cell immunity while preserving MHC-I for preventing host NK killing. We screened a panel of T cell second signaling molecules and found that knocking out some of the molecules significantly reduced the stimulation to T cells. Importantly, the allogeneic cells with a simultaneous knockout of molecules M1 and M2 would not elicit T cell immunity, which was comparable to MHC-I KO. We then applied the edits on GMP-grade human iPSCs and demonstrated that M1/M2 double knockout did not impact NK differentiation and had robust resistance towards human T cells and NK cells *in vitro.* Taken together, we identified that destruction of T cell synapse formation could render iPSCs-derived NK cells hypoimmunogenic in vitro. We anticipate that this could be a simple and useful asset to greatly enhance cell therapy efficacy.

634 CAR-Treg Cells Differentiated *In Vivo* by Transduced Hematopoietic Stem Cells: A New Approach to Treatment of Autoimmune Disorders

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Autoimmune diseases such as multiple sclerosis (MS) are commonly treated with non-specific anti-inflammatory drugs. Autologous hematopoietic stem cell (HSC) transplantation shows efficacy in some forms of MS by providing an "immune reset" function, even though it does not specifically target autoimmune-mediated neurodegeneration. Several studies showed that adoptive transfer of regulatory T cells (Tregs) expressing chimeric antigen receptors (CAR) directly addresses autoimmunity in murine models of MS. Though promising, this approach faces limitations regarding Treg stability *in vivo* and long-term efficacy. We are developing a new approach to treat autoimmune conditions, by enabling Treg therapy through transplantation of autologous HSCs genetically modified with a lentiviral vector to express an antigen-specific CAR under Treg-restricted transcriptional control. Genetically modified HSCs have the potential to generate stable, long-lived antigen-specific CAR-Tregs *in vivo* (Fig. 1).

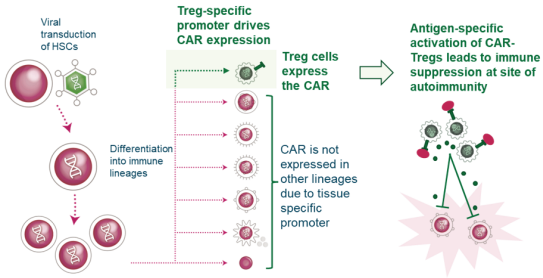

Figure 1. HSC-derived CAR-Tregs. An antigen-specific CAR is selectively expressed in differentiated Treg cells due to Treg-restricted transcriptional regulation. CAR-Tregs can be specifically activated by (auto)antigens, leading to local suppression of autoreactive responses.

Candidate transcriptional regulatory elements derived from the human FOXP3 gene were characterized in different combinations for activity and specificity in a lentiviral vector construct both *in vitro,* in primary murine and human T cells, and *in vivo,* upon transplantation of transduced HSCs into lethally conditioned mice. FOXP3-derived synthetic promoters are preferentially activated in Tregs in both contexts. In parallel, CARs were designed to induce Treg functions upon engagement with specific target antigens, leading to secretion of the immunosuppressive cytokine IL-10 from transduced murine and human Tregs upon antigen stimulation *in vitro*. To demonstrate the concept in vivo, murine lineage negative hematopoietic stem/progenitor cells (*Lin*$^-$ HSPCs) were transduced with lentiviral vectors to express CAR under the control of the FOXP3-derived synthetic promoter and transplanted into recipient mice. The CAR was efficiently expressed in Tregs 3 months post-transplant (Fig. 2A), with no impact on normal Treg development, phenotype or function. *Ex vivo* ligand-specific activation of CAR-Tregs led to enhanced Treg function with increased expression of CD25 (Fig. 2B) and IL-10 secretion (Fig. 2C).

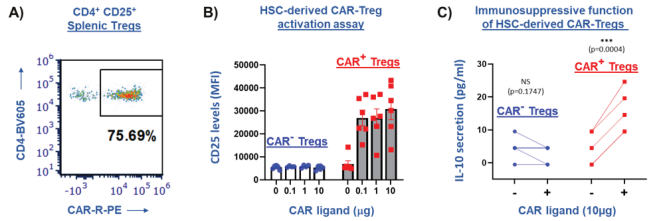

Figure 2. HSC-derived CAR-Tregs express a functional CAR *in vivo*. Mice were transplanted with *Lin*$^-$ HSPCs transduced with LVs designed to express an antigen-specific CAR under the control of a Treg-restricted promoter. Splenic Tregs successfully express the CAR **(A)** and respond to ligand-specific stimulation by increasing CD25 levels **(B)** and IL-10 secretion **(C)**.

These data represent a first preclinical proof of concept for the generation of functional CAR-Tregs *in vivo* from genetically engineered HSCs, and a first step towards the development of potential lifelong treatments for chronic autoimmune disorders.

635 Regulated Expression of GATA1 as a Gene Therapy Cure for Diamond-Blackfan Anemia

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Diamond-Blackfan anemia (DBA) is a bone marrow failure disorder characterized by severe anemia due to impaired red blood cell production, and has limited treatment options. More effective, safer, and permanent cures for DBA are desperately needed, but few drugs are in active development. DBA is genetically heterogenous, caused by haploinsufficiency in 1 of at least 26 different ribosomal protein (RP) genes that alters the translation of select transcripts, most notably the erythroid master regulator GATA1. Constitutive expression of GATA1 in hematopoietic stem cells (HSCs) from patients with DBA rescues the erythroid differentiation defects *in vitro* but impairs long-term (LT-) HSC function. Gene therapy is an attractive strategy to achieve a cure for DBA, but a traditional gene therapy approach that overexpresses a functional copy of a mutated gene requires the development and validation of dozens of gene therapy vectors, each with a copy of one of the mutated DBA genes. Here, we report a unified gene therapy strategy, using developmentally regulated expression of GATA1 to be curative in most, if not all, patients with DBA, regardless of the underlying disease-causing mutation. We sought to design a GATA1 gene therapy vector that incorporates into the genome of undifferentiated LT-HSCs but only drives robust GATA1 expression in committed erythroid progenitors. To achieve this, we identified chromatin regions upstream of *GATA1* that are accessible only in differentiating erythroid cells, but not in HSCs. Using those selectively accessible regions concatenated together, we constructed a human GATA1 enhancer (hG1E) element and used it to drive GATA1 and/or GFP expression from a lentiviral backbone in HSCs collected from healthy human donors. We observed very low transgene expression in bulk HSCs and essentially no expression

in the subpopulation of LT-HSCs with no impairment in stem cell function *in vivo*. However, more than 80% of committed erythroid cells demonstrated high levels of transgene expression, confirming the fidelity of lineage-restricted expression from the hG1E-GATA1 vector. To examine whether lineage-restricted GATA1 expression confers a functional correction of the erythroid maturation block in DBA, we used CRISPR editing to recapitulate *RPS19* haploinsufficiency in human HSPCs. In *RPS19* edited samples treated with a control GFP virus, we observed a progressive selection against *RPS19* edited cells as they underwent erythroid differentiation. In contrast, *RPS19*-edited and hG1E-GATA1 treated samples had a more than 10-fold higher preservation of *RPS19* edits in the bulk population and in erythroid colonies derived from single progenitor clones, indicating that regulated GATA1 expression can overcome *RPS19* haploinsufficiency. To validate these results, we treated primary bone marrow samples from 10 DBA patients with varied genotypes, representing almost 75% of all DBA patients with a defined genetic cause. Control treated cells showed impaired erythroid differentiation with skewing down the myeloid and megakaryocyte lineages. In contrast, regulated GATA1 expression from the hG1E-GATA1 vector in DBA patient cells led to a restoration of erythroid differentiation with significantly increased erythroid output. Additional preclinical safety and *in vivo* efficacy studies are ongoing. Collectively, our preclinical data demonstrate that lineage-restricted expression of GATA1 is sufficient to rescue the impaired erythroid differentiation in DBA regardless of the underlying genetic lesion. These results will set the stage for the first in-human gene therapy trial for DBA.

636 Development of KYV-101, a Novel CD19 CAR-T Cell Therapy for the Treatment of B Cell-Driven Autoimmune Diseases

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Background: A significant unmet medical need remains in the treatment of relapsed and/or refractory B cell-driven autoimmune diseases. The presence of autoantibodies is a hallmark of such diseases and implicates dysregulated B cell function in their pathogenesis. The central role of B cells in these diseases is also supported by the presence of increased numbers of B cells in diseased tissues and the efficacious responses reported with biological therapies that target B cells. KYV-101 is an autologous CD19 chimeric antigen receptor (CAR)-T cell therapy that depletes pathogenic B cells. Importantly, the CD19 CAR utilized in KYV-101 was previously tested in B-cell lymphoma patients and demonstrated efficacy with an improved safety profile (1). Since CD19 CAR-T cells target and lyse B cells in both circulation and tissues, a more complete depletion of autoreactive B cells is expected with KYV-101 than biological therapies, resulting in better disease control and clinical remission than the current immunotherapies. **Methods:** Autologous $CD4^+$ and $CD8^+$ T cells were enriched from healthy donors (HD) and systemic lupus erythematosus (SLE) patients. KYV-101 CAR T cells were generated by transducing T cells with a lentiviral vector encoding a CAR comprised of a fully human single-chain variable fragment (scFv) CD19-targeting domain, a CD8a hinge and transmembrane domain, a CD28 cytoplasmic costimulatory domain, and a CD3z cytoplasmic domain. The binding affinity and specificity of the anti-CD19 scFv for recombinant human CD19 protein and $CD19^+$ cells were evaluated in vitro in a set of in BioLayer Interferometry, flow cytometry and cell-based protein microarray screening studies. KYV-101 CAR-mediated and CD19-dependent activity was also monitored in vitro and in vivo in a set of cytotoxicity, cytokine release, proliferation, and tumor growth studies, in response to either a $CD19^+$ target cell line, autologous, patient-derived primary $CD19^+$ B cells, or a $CD19^-$ target cell line. **Results:** The anti-CD19 scFv used in KYV-101 bound with high affinity to both recombinant human CD19 protein and $CD19^+$ NALM6 cells. Moreover, the anti-CD19 scFv was specific for CD19 as no other specific interactions were observed in the cell-based protein microarray screen. KYV-101 demonstrated CAR-mediated and CD19-dependent activity in vitro. KYV-101 generated from HDs or SLE patients induced greater and dose-dependent cytotoxicity of both the human $CD19^+$ cell line (NALM6) and autologous, patient-derived primary B cells than their respective untransduced control T cells. Moreover, an effector cell dose-dependent increase in the production of cytokines such as IFNγ was also observed following co-culture. In contrast, no differences in cytotoxicity nor cytokine production were observed for KYV-101 or untransduced control T cells co-cultured with $CD19^-$ K562. Furthermore, KYV-101 generated from HDs or SLE patients showed enhanced proliferation when co-cultured with the $CD19^+$ NALM6 cells and autologous, patient derived primary B cells compared to the untransduced control T cells co-cultured with either $CD19^+$ or $CD19^-$ cells. Importantly, KYV-101 also demonstrated CAR-mediated mechanism of action in vivo as transferring KYV-101 into NSG mice inoculated subcutaneously with $CD19^+$ NALM6 tumor cells resulted in complete tumor ablation at doses of at least $3x10^6$ CAR^+ cells/mouse. **Conclusion:** KYV-101 generated from HDs and autoimmune disease patient lymphocytes demonstrates high affinity and specificity to human CD19 as well as CAR-mediated and CD19-dependent activity in vitro and in vivo against $CD19^+$ cell lines and/or autologous, patient-derived primary B cells. KYV-101 thus represents a novel therapeutic option for the depletion of pathogenic B cells in autoimmune patients. **References:** [1] Brudno JN, et al., Nat Med, 2020, 26(2):270-280

637 High-Throughput Screening of Polymers for *In Vivo* Hematopoietic Stem Cell Base Editing in a Novel Fanconi Anemia Murine Model

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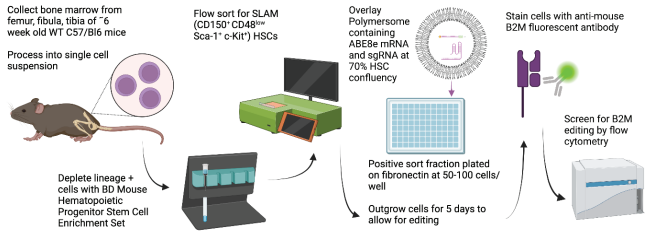
Allogeneic hematopoietic stem cell transplant (HSCT) is the current standard of care for bone marrow failure (BMF) associated with Fanconi anemia (FA). FA patients face unique challenges including difficulty sourcing matched donors, increased sensitivity to HSCT pre-conditioning regimens, and high risk of malignancy. The Spanish

founder mutation (1:64 carrier frequency) of FA arises from a single C→T nucleotide substitution in exon 4 of the *FANCA* gene and forms a premature stop codon that results in a truncated, non-functional FANCA protein. Restoration of the *FANCA* coding sequence to a functional variant in hematopoietic stem and progenitor cells (HSPCs) can reverse the effects of BMF. Recently published work by our group and that of Siegner et al. demonstrated that adenine base editor (ABE) can correct the Spanish founder mutation and restore protein function *in vitro*, prompting us to pursue novel approaches for *in vivo* correction. To facilitate these efforts, we generated a novel murine model in which exon 4 of the murine *Fanca* locus was replaced with human wild-type or mutant *FANCA* sequence, enabling the utilization of our validated human gene editing reagents in a clinically relevant animal model. Numerous methods for targeted *in vivo* delivery of genome editing reagents are under investigation, however, polymer-based delivery has the advantage of scalability and flexible modular design. We are performing high-throughput screening of polymer complexes for delivery to HSPCs, as shown in Figure 1. Initial screens will be carried out on primary C57BL/6 murine HSPCs *in vitro* by delivering ABE8e mRNA and sgRNA targeting β2-microglobulin (*B2m*), with functional editing of target HSPCs determined by flow cytometric quantitation of surface B2m expression. High-performing polymers will be combined with ABE8e mRNA and therapeutic sgRNA and evaluated in HSPCs isolated from FA mice. Following confirmation of genetic and functional correction, HSPCs will be transplanted into lethally-irradiated FA mice and subsequently monitored over several weeks for immune reconstitution by peripheral blood mononuclear cell collection and flow cytometry. In pursuit of a more accessible and cost-effective therapy for FA, we will administer the validated polymer-ABE8e complexes to FA mice *in vivo* alongside an adenovirus control delivering the same cargo. If delivery to HSPCs is successful, we expect to recover mitomycin C (MMC)-resistant cells from peripheral blood and marrow within 16 weeks. At the endpoint, bone marrow will be collected for genomic sequencing and flow-sorted to isolate Lin- c-Kit+ Sca1+ HSPCs for further study. To confirm restoration of function, we will perform Western blot for FANCA, culture these cells in the presence of MMC to determine their ability to perform interstrand crosslink repair, and measure the monoubiquitination of FANCD2 by Western blot. This work will validate the *in vivo* correction of the *FANCA* Spanish founder mutation and lay the groundwork for non-viral gene therapy for FA patients.

Figure 1: Schematic representation of initial polymer screening.



638 Demonstration of Human Factor VIII Expression and Activity Following Single and Repeat Dosing of a Non-Viral Integrating Gene Therapy

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The current first-line treatments for Hemophilia A patients are clotting factor replacement and/or bi-specific antibodies. These treatments require continuous, lifelong infusions, yet many patients have breakthrough bleeds. There remains a high unmet need for safe, effective, and durable therapies. We evaluated the potential of a liver-directed non-viral in vivo gene insertion approach using the super piggyBac® DNA insertion system to produce stable human FVIII (hFVIII) expression in mice. Unlike conventional AAV-based gene therapy approaches, our platform enables delivery of large transgenes, the ability to stably and safely integrate the therapeutic transgene into the genome, and the potential for re-dosing to titrate to target FVIII activity. We first developed a dual liver-tropic nanoparticle system that entails one lipid nanoparticle (LNP) encapsulating the super piggyBac transposase (SPB) formulated as mRNA (LNP-SPB), and a second LNP encapsulating the transposon formulated as plasmid DNA and comprising the hFVIII open reading frame, a promoter, and super piggyBac inverted terminal repeats (LNP-hFVIII).To validate the durability of this system, mice were dosed intravenously with the two individual LNP formulations, each optimized for either the delivery of the SPB mRNA or the therapeutic hFVIII transposon DNA cassette. We observed durable and dose-dependent hFVIII expression in these models of dividing liver significantly above normal levels. To further support the concept of re-dosing, we treated wild type mice up to 4 times and observed a dose-proportionate increase in hFVIII antigen levels after each administration. We subsequently evaluated a co-encapsulated LNP formulation, comprising both SPB mRNA and transposon plasmid DNA in the same liver-tropic nanoparticle in juvenile Hemophilia A mice and observed a ~50% increase in hFVIII antigen expression compared to the dual nanoparticle approach. No evidence of persistent liver enzyme elevation was observed following treatment. To validate the co-encapsulated nanoparticle approach, we performed a study in a mouse model of severe hemophilia A (FVIII knock-out) tolerized to prevent immune response against human FVIII. Following a single dose in adult mice, we observed ~30% hFVIII expression sustained over the duration of the study. The integration profile was assessed, and no insertions were found within tier 1 or tier 2 COSMIC tumor suppressor genes. Our results demonstrate the capabilities of the super piggyBac DNA insertion system and non-viral approach in providing stable FVIII transgene expression through

genomic integration, along with the potential for redosing. These data provide proof-of-principle toward developing a potential functional cure for Hemophilia A.

639 A New Liver-Humanized Mouse Model for the Study of Hepatitis B Virus and Human Hepatic Gene Editing

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Liver-humanized mice are a powerful tool for drug metabolism and pharmacokinetics studies, for the study of hepatotropic infectious agents such as hepatitis B virus (HBV), hepatitis C virus (HCV), and malaria, and for the study of hepatic human gene editing *in vivo*. However, the commercial cost and complexity of existing liver-humanized mouse models including uPA-SCID, TK-NOG, and FRG mice has limited their widespread use in academic research. To establish a cost-effective model that we could use for the study of HBV, hepatic gene transfer, and human hepatic gene editing, we evaluated the recently described liver humanized NSG-PiZ mouse model that is relatively inexpensive and easy to establish. Since HBV is extremely species-restricted, we first challenged liver-humanized NSG-PiZ mice with HBV, a pathogen of major public health importance that is largely incurable once a chronic infection is established, to determine whether they are also permissive to HBV replication. After administration of a genotype D clinical isolate, chronic infections lasting at least 6 months could be established, and we were able to serially passage virus through 3 generations of mice without loss of viral fitness. HBV selectively replicated in hCK18+ human hepatocytes and HBV+ mice had viral loads and HBsAg levels in blood comparable to those seen in patients not receiving antiviral therapy. Importantly, livers of HBV+ mice harbor covalently closed circular DNA (cccDNA), the template for viral replication, enabling the study of curative antiviral therapies that target cccDNA. We next showed that HBV+ liver-humanized NSG-PiZ mice can be used to evaluate antiviral therapies by treating HBV+ mice with the reverse transcriptase inhibitor entecavir, a frontline antiviral agent used to treat HBV+ patients. Over 4 weeks of entecavir therapy, viral loads decreased by approximately 3 logs compared to untreated mice, then rebounded after entecavir withdrawal. We finally showed that HBV- and HBV+ human hepatocytes can be efficiently transduced by AAV-vectors *in vivo*, which should enable the delivery of antiviral gene therapeutics or gene editing reagents that target HBV or human hepatic genes. HBV+ NSG-PiZ mice were administered AAV vectors packaged with the hepatotropic AAV3b and AAV.LK03 vector capsids and gene transfer was analyzed at 4 weeks post vector administration. While both vector capsids mediated transduction of both mouse hepatocytes and human hepatocytes, AAV.LK03 preferentially transduced human hepatocytes with high efficiency, which should enable the study of curative gene therapies targeting chronic HBV or hepatic human gene editing. In summary, we have established a robust and cost-effective model for HBV that can be used as an alternative to existing models in the study of established and new therapeutic approaches targeting chronic HBV infections. Our model may also be used to study human hepatic gene editing *in vivo* since human hepatocytes can be readily targeted by AAV vectors.

640 Transcriptomic Profiling of Glycogen Storage Disease Type IX γ2 Mouse Liver and Matched Plasma to Identify Biomarkers for Gene Therapy

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The understanding of gene expression pathway changes and the identification of potential biomarker expression can further our understanding of gene interactions, provide critical exploratory endpoints for gene therapy treatment, and support clinical development. Availability of this data for rare diseases is limited by the relatively small investment in research on the disease natural history. Glycogen storage disease (GSD) type IX is caused by deficiency in phosphorylase kinase (PhK), the enzyme that initiates glycogenolysis. PhK in the liver is comprised of four subunits: α2, β, γ2, and δ. Biallelic pathogenic variants in the gene encoding the γ2 subunit (PHKG2) causes GSD IX γ2 in which affected patients are unable to breakdown glycogen in the liver and exhibit fasting ketotic hypoglycemia, hyperlipidemia, altered LDL/HDL cholesterol ratios, and progressive liver fibrosis with risk for cirrhosis. We identified metabolic pathway changes and potential biomarkers for GSD type IX γ2 using transcriptomic profiling of a Phkg2 knock-out (KO) mouse model during a critical period of disease pathophysiology development. In this study, we obtained WT and KO mouse liver and plasma samples at 4, 6, 8, and 12 weeks of age. Following mRNA and small RNA extraction from liver and plasma, respectively, we prepared libraries using an Illumina Stranded mRNA kit and PerkinElmer Nextflex small RNA-Seq v4 kit and sequenced these on an Illumina NextSeq 2000. Our results reveal that genotype explains the highest proportion of transcriptomic variation, followed by the age of the mouse, for both the liver mRNA and plasmid small RNA samples. In the liver, there are striking changes in the expression levels of key enzymes involved in glycogen synthesis and glycogenolysis including Gys2 and Pygl, respectively. Markers of liver fibrosis and inflammation, such as Smad7, Ccl2, and Itgb5, are increased in the KO livers relative to WT, specifically at later time points. The expression of several lipid metabolic enzymes also differed in the livers of WT and KO animals. These results are mirrored in our analysis of matched plasma samples, in which we observed significant differences in the expression of liver-specific microRNAs in plasma from KO compared to WT animals, including the well-studied miR-122-5p, which has been associated with an adverse metabolic profile in the liver. This study provides the first transcriptomic analysis of a GSD IX γ2 mouse model and allows for the identification of potential hepatic metabolic changes and biomarkers that vary across the natural course of the disease in both liver and plasma, improving our understanding of disease mechanisms. These biomarkers will be critical for assessing the impacts of therapy

interventions on liver fibrosis and metabolic function restoration in the GSD IX γ2 mouse model and will be pursued as exploratory endpoints for future clinical studies in patients treated with gene therapy.

641 Co-Formulation of Peptide Dendrons with Liposomes for Liver Delivery of mRNA and DNA

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Nucleic acid therapies have the potential to treat or even cure a wide range of diseases through knockdown, expression, and/or editing of target genes. However, achieving efficient, safe, and selective delivery of nucleic acid cargoes remains a challenge. In this work, we investigated nanoparticle-based methods to deliver DNA and mRNA to the liver. Proprietary lysine-based peptide dendrons (PDs) were used to condense DNA or mRNA into nanoparticles in the presence of DOPE/DOTMA liposomes (Lipofectin®), resulting in a peptide-lipid coformulation. PDs were further modified with N-Acetylgalactosamine (GalNAc) ligands to enhance delivery to liver *in vivo* via the asialoglycoprotein receptors (ASGPR) expressed on hepatocytes. Peptide dendrons and Lipofectin® were combined at varying ratios before mixing with nucleic acid and allowed to self-assemble into nanoparticles in aqueous buffer. The resulting nanoparticles were 150-200 nm in diameter with spherical morphology. DNA and mRNA cargoes encoded for luciferase were used to track expression in mice by IVIS bioluminescence imaging following tail vein injection. Peptide dendrons and Lipofectin® were shown to act synergistically, significantly enhancing liver expression of DNA and mRNA over peptide dendrons or liposomes alone. Additionally, we observed an increase in transfection with increasing GalNAc density, showing that transfection was driven by specific interactions between GalNAc targeting ligands and ASGPR expressed on hepatocytes. This platform also showed differences in expression kinetics for mRNA and DNA cargoes. Cargo selection significantly impacted expression kinetics; mRNA showed strong transient liver expression for 48 hours, while DNA expression was sustained for over 20 days. Taken together, these results show that targeted peptide dendron-liposome co-formulations are a promising and versatile platform for nucleic acid delivery to liver.

642 Liver-Directed Gene Therapy for Ornithine Aminotransferase Deficiency

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Gyrate atrophy of choroid and retina (GACR) is a chorioretinal degeneration caused by pathogenic variants in the gene encoding ornithine aminotransferase (OAT), an enzyme mainly expressed in liver. Affected patients have increased ornithine concentrations in blood and other body fluids and develop progressive constriction of vision fields leading to blindness. Current therapies are unsatisfactory and better treatments are highly needed. In two mouse models of OAT deficiency that recapitulates biochemical and retinal changes of GACR, we investigated the efficacy of an intra-venously injected serotype 8 adeno-associated (AAV8) vector expressing OAT under the control of a hepatocyte-specific promoter. Following injections, OAT-deficient mice showed reductions of ornithine concentrations in blood and eye cups compared with control mice injected with a vector expressing green fluorescent protein. AAV-injected mice showed improved electroretinogram response and partial restoration of retinal structure up to one-year post-injection. In summary, hepatic OAT expression by AAV8 vector was effective at correction of hyperornithinemia and improved function and structure of the retina. In conclusion, this study provides proof-of-concept of efficacy of liver-directed AAV-mediated gene therapy of GACR.

643 *In Vivo* Gene Therapy for Lysosomal Acid Lipase Deficiency

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Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive disorder caused by mutations in the *LIPA* gene. LAL enzyme is involved in lysosomal hydrolysis of cholesterol esters and triglycerides and its deficiency triggers fats accumulation leading to multi-organs failure. The most severe form of LAL-D (Wolman disease, WD; ≤5% residual

LAL activity) affects ~1/300,000 live births and, if not treated, results in premature death within the first year of life due to denutrition and hepatic insufficiency. Enzyme replacement therapy (ERT) is the only available supportive treatment and consists in weekly systemic injection of recombinant LAL. Although life-saving, ERT is not curative and is associated with the risk of developing neutralizing antibodies that can decrease treatment efficacy. In order to develop a curative treatment for WD, we first characterized a new *lal*$^{-/-}$ mouse model and then assessed a gene therapy treatment based on the *in vivo* administration of recombinant AAV vector encoding human LAL. The full *lal*$^{-/-}$ mouse model was generated by deleting the exon 4 of the *LIPA* gene. We confirmed the loss of LAL protein and activity and the accumulation of cholesterol and triglycerides in liver, spleen, and jejunum. Lipid overload in the liver leads to an increase in liver transaminase and a change in the blood lipid profile, in particular HDL decrease. Moreover, *lal*$^{-/-}$ mice display a dysregulation in haematological compartment, with an inflammatory profile associated with an increase of granulocytes, monocytes/macrophages and platelets and a decrease of lymphoid population. We are currently investigating the effect of LAL deficiency on cellular metabolism and mitochondria. For gene therapy, we decided to target the liver by systemically injecting 3-month-old *lal*$^{-/-}$ mice using the AAV-8 serotype encoding the hLAL gene under the control of hepatocyte-specific human α1-antitrypsin (hAAT) promoter. Modified hepatocytes would both produce LAL to correct their own metabolic impairment and secrete the enzyme in bloodstream to cross-correct affected tissues. Starting from two weeks after injection, we restored LAL expression/activity in plasma and we observed a therapeutic effect in term of haematological parameters, decrease of hepatic transaminase and normalization of HDL profile. At 3- and 10-months post-injection, we sacrificed mice, and we analysed tissue correction by histological staining, cholesterol/triglycerides quantification, and measurement of LAL enzymatic activity. Assessment of mitochondrial function is currently under investigation. To decrease vector dose and maximize the therapeutic benefit of our *in vivo* gene therapy strategy, we engineered *LIPA* cDNA to improve LAL expression and secretion in cells (~10x) by optimizing the codon usage and changing its signal peptide sequence and we are currently evaluating its therapeutic benefit *in vivo*. Overall, our *in vivo* gene therapy strategy appears to be an effective approach to achieve stable LAL expression to correct WD disease phenotype in a relevant *lal*-/- mouse model. Therefore, the development of such AAV-based gene therapy would offer a curative treatment of LAL-D patient improving their life expectancy and quality.

644 Selective Expansion of Transplanted Hepatocytes Corrects a Mouse Model of Phenylketonuria

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Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism caused by a deficiency in the enzyme phenylalanine hydroxylase (PAH). PAH is responsible for the metabolism of phenylalanine (Phe) to tyrosine in hepatocytes. In the absence of PAH, highly elevated Phe levels in the blood lead to severe neurological symptoms unless patients maintain a highly restricted diet. Several AAV gene therapy approaches are in development for PKU. However, these treatments are predicated to be of limited efficacy for pediatric patients as cell division in the growing liver leads to episome loss. An alternative strategy for permanent correction of PKU is hepatocyte transplantation, a cell therapy method wherein donor hepatocytes are delivered into circulation where they engraft in the recipient liver and support liver function. A limitation of this method is the low rate of engraftment of the delivered cells. This problem could be overcome by selectively expanding the transplanted cells until they make up enough of the liver to correct blood Phe levels. We have previously reported a system to expand gene edited hepatocytes in vivo using the common fever medication acetaminophen (APAP). This is accomplished by knocking out the gene cytochrome P450 reductase (Cypor), which is required for the metabolism of APAP into its hepatotoxic metabolite. Thus, when the liver is treated with high levels of acetaminophen, Cypor deficiency confers a survival advantage and these cells expand to repopulate the liver. We have previously presented proof-of-concept for the correction of PKU using this selection system, and here we report robust correction with long term follow-up. Primary hepatocytes were isolated from a wildtype mouse and treated in vitro with Cas9 ribonucleoprotein complexes to create a knockout of the Cypor gene. Hepatocytes were delivered to *Pah*-deficient mice, which were subsequently treated with an APAP-containing diet. Correction of blood Phe levels to within the physiological range was achieved in both male and female animals, and in multiple strains of *Pah*-deficient mice. This correction was sustained for >250 days after discontinuing APAP treatment. Upon harvest, clonal expansion of Cypor-deficient hepatocytes was observed in APAP-treated mice. By comparison, animals that received a hepatocyte transplant without APAP selection showed only rare transplanted cells equivalent to <1% of the liver mass and no reduction in blood Phe. Analysis of insertion/deletion mutations at the Cypor-targeted guide RNA locus in the APAP-treated corrected animals indicated approximately 15% Cypor deficient hepatocytes, corresponding to the threshold of *Pah*-expressing hepatocytes required to correct the PKU phenotype. Compared to a known maximum of >40% Cypor-deficient hepatocytes that can be achieved with the APAP selection system, this indicates that a majority of susceptible hepatocytes retain Cypor activity. This mitigates concerns that partial Cypor deficiency will lead to unintended consequences. To further examine the effects of partial Cypor deficiency, metabolism of a Cypor-metabolized drug will be assessed in selected animals. The data presented here indicate that hepatocyte transplantation with the APAP selection system is a potential therapeutic for the complete, long-term correction of PKU. This strategy has potential applicability to a variety of inborn errors of metabolism.

645 Versatile Knock-Out and Transgenic Murine Models of *PCCB* Deficiency Enable the Testing of Systemic AAV Gene Delivery as a Treatment for Propionic Acidemia

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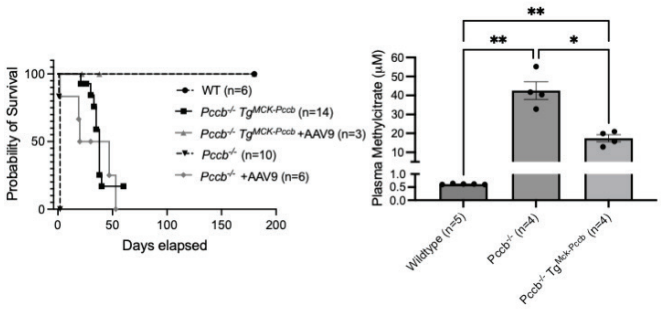
Propionic acidemia (PA) is rare autosomal recessive metabolic disorder caused by defects in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). The PCC enzyme is composed of six nuclear encoded α- and six β-subunits with causative variants in either of the *PCCA* or *PCCB* genes found at equal frequencies.

Individuals with PA can suffer from lethal metabolic instability and cardiomyopathy despite medical management, which has provided a strong rationale to develop gene therapy as a possible treatment option for patients. There are no publications which describe murine models of PCCB deficiency or the development of gene therapy for this subtype of PA. We have therefore developed new murine models of PCCB deficiency, one severe and another milder, and tested AAV gene therapy as a treatment in the mice. CRISPR-Cas9 gene editing targeting the 14th exon of the murine *Pccb* gene was used to introduce a 4 base pair deletion (c.1492_1495delCCAG), predicted to result in a frameshift stop mutation (p.A489Pfs*1). Mice homozygous for this mutation ($Pccb^{-/-}$) display a neonatal lethal phenotype with 100% of the affected mice perishing in the first 72 hours of life and manifest massively elevated plasma 2-methycitrate levels in the plasma. In a proof-of-concept study, $Pccb^{-/-}$ mice treated at birth with 6.6e14 vg/kg of AAV9.CAG.PCCB, delivered by retroorbital injection, had a significant increase in survival in comparison to untreated mutant controls ($p<0.01$). Due to the severe lethality displayed by the $Pccb^{-/-}$ mice, we designed a germline transgene to express the murine *Pccb* cDNA under the control of a muscle specific promoter ($Tg^{MCK\text{-}Pccb}$) and through breeding generated $Pccb^{-/-}$ $Tg^{MCK\text{-}Pccb}$ mice in order to assess clinical and biochemical effects on the severe PA phenotype of the $Pccb^{-/-}$ mice. The resultant $Pccb^{-/-}$ $Tg^{MCK\text{-}Pccb}$ mice display increased survival, growth retardation, and moderate elevations of plasma methylcitrate in comparison to the $Pccb^{-/-}$ mice without the transgene. To test the utility of the $Pccb^{-/-}$ $Tg^{MCK\text{-}Pccb}$ mice to evaluate new therapies, we treated mutant mice at 30 days with a dose 1e14 vg/kg of AAV9.CAG.PCCB delivered by retroorbital injection and observed a significant increase in survival in comparison to untreated controls ($p<0.02$). These new murine models of PA span the clinical spectrum of severity, manifest cardinal clinical and biochemical features of PCCB deficiency, and can be used to test the effects of new genomic therapies for PA, including AAV9 gene therapy.



646 A Mouse Model Resource to Test Gene and Cell-Based Therapies for Peroxisomal Disorders

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Peroxisomes are metabolic organelles that serve as a central hub of cell signaling pathways and play essential roles in the development and functions of all organ systems. Peroxisome dysfunction is causally responsible for a group of rare monogenic disorders including X-linked adrenoleukodystrophy, Zellweger spectrum disorder, D-bifunctional protein deficiency, and adult Refsum disease. They also contribute to the pathophysiology of a diverse group of common disorders including diabetes, cancer, and Alzheimer's disease as well as a host of infectious diseases. They contact and exchange materials with other organelles and are thus critical for cellular metabolism. Despite their relevance to numerous facets of human health and disease, the limited number of well-annotated publicly available mouse models and immunological resources required to investigate various aspects of peroxisome biology and test gene and cell-based therapies has hindered the biomedical research community. Moreover, the impact of many existing mouse models has been lessened since they often are placed on suboptimal genetic backgrounds or are not readily available to the public. Here, we have established the Mouse Peroxisome Research Resource (MPRR) that will provide a central resource for mouse models and monoclonal antibodies for basic and translational research relevant to peroxisome biology and disorders caused by peroxisome dysfunction. The MPRR is a community-driven effort that leverages a world-leading knowledge of mouse genetics, gene editing, and monoclonal antibody production capability as well as expertise in model development and disease model repositories to accelerate the creation, distribution, and proper use of high-impact mouse models and monoclonal antibody reagents. The MPRR will ensure that all deposited mouse models are on standardized genetic backgrounds to control for the presence of genetic modifiers. These strains will be made available as well-annotated resources with as few legal restrictions as possible. The MPRR will also produce novel high-priority mouse models with defined genotypes on standardized genetic backgrounds, cryopreserve them, and distribute them to the public. Moreover, the MPRR will also assist in the targeted phenotyping of these models, including measurement of relevant peroxisomal metabolite levels. Furthermore, the MPRR will produce and publicize and distribute validated monoclonal antibody reagents for peroxisome research, including characterizing relevant mouse models. Overall, the MPRR will dramatically increase the number of available mouse models and monoclonal antibody reagents based on community-driven priority and accelerate preclinical testing of rationally designed cell and gene-based therapeutic interventions for disorders caused by peroxisome dysfunction.

647 Therapeutic Efficacy of VTX-801 in Advanced Stage of Wilson's Disease in Mice

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Wilson's disease (WD) is a rare disorder of copper metabolism inherited in an autosomal recessive manner. It is due to missense mutations in *ATP7B*, a copper-transporting P-type ATPase, that lead to hepatic, neurologic, or psychiatric symptoms. Left untreated, the condition progresses to severely debilitating complications and death. VTX-801 is a recombinant adeno-associated vector (rAAV) carrying a shortened version of the human *ATP7B* gene. VTX-801 has demonstrated to provide long-term correction of copper metabolism and ceruloplasmin levels, preservation of liver integrity and function when administered at early stage of the disease in WD mice. Based on these results, GATEWAY, a phase I/II clinical trial was initiated to evaluate VTX-801 in adult patients with WD. To extend previous results, we assessed the therapeutic efficacy and safety of VTX-801 in advanced stage disease WD mice by administering a dose defined as therapeutic in younger animals.[1] Thus, a single dose of VTX-801 was injected to 12-, 16- & 20-week-old WD mice, presenting with progressive liver abnormalities including inflammatory foci, single cell necrosis and hepatocyte hypertrophy. VTX-801-treatment outcome was assessed 6 months later by measuring copper metabolism parameters, liver injury and function, hematological parameters, liver histology, and liver transgene expression and transduction. Although most of pharmacodynamic effects of VTX-801 observed in younger mice were retained -urinary copper excretion rate remained relatively low, liver function (albumin, cholesterol and glucose) improved and tissue (brain, kidney and liver) copper content reduced compared to untreated animals-, certain liver histological features such as fibrosis were not fully reversed in older animals. The quantification of viral genome copies and transgene expression in livers of WD mice treated at 12 weeks of age confirmed the persistence of the viral genomes and the sustainability of transgene expression at time of sacrifice. However, lower transduction efficiency might explain the reduction in therapeutic effect observed in older animals. These results demonstrate that VTX-801 can slow down Wilson's disease progression by improving major pathological features of the disease and may represent a promising treatment also for late stage of this condition in WD patients.

1 Murillo O, et al. Liver Expression of a MiniATP7B Gene Results in Long-Term Restoration of Copper Homeostasis in a Wilson Disease Model in Mice. Hepatology. 2019 Jul;70(1):108-126. doi: 10.1002/hep.30535. Epub 2019 Mar 20. PMID: 30706949.

648 Codon-Optimization Enhances the Expression/Secretion of rSGSH, and Functional Benefits to Treat MPS IIIA in Mice via Systemic scAAV9-h*SGSH*^{op} Gene Delivery

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Mucopolysaccharidosis (MPS) IIIA is a devastating neuropathic lysosomal storage disease caused by autosomal recessive defects in sulfoglucosamine sulfohydrolase (SGSH). No treatment is available for MPS IIIA. To address the unmet need, we developed a new scAAV vector to deliver the codon-optimized human SGSH gene (h*SGSH*^{op}), driven by a truncated mini CMV (mCMV) promoter. *In vitro* studies showed that the scAAV-h*SGSH*^{op} vector enhanced the expression and more importantly the secretion of rSGSH. We tested the vector in 1-2m-old MPS IIIA mice with a single IV injection of scAAV9-h*SGSH*^{op} vector at doses 4e12vg/kg - 8e13vg/kg. The results showed that the vector treatments led to in rapid and persistent restoration of SGSH activity and the clearance of lysosomal storage pathology throughout the CNS, peripheral nervous system (PNS) and broad peripheral tissues. Importantly, behavior testing in Morris water maze at 7m pi showed significant improvement in learning and swimming ability in the vector treated MPS IIIA mice. Further, the majority of MPS IIIA mice treated with the vector survived within the normal life span. These data indicate the functional correction of neurological manifestation. The vector treatments did not result in any detectable adverse events or systemic toxicity in MPS IIIA mice. In this study, we have developed a new effective and safe gene replacement therapy product, supporting the promising clinical potential of systemic scAAV9-h*SGSH*^{op} gene delivery for treating MPS IIIA in humans.

649 Hematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis Type IIIC

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Mucopolysaccharidosis type IIIC (MPSIIIC) is a severe progressive childhood neurodegenerative disease caused by loss-of-function of the transmembrane lysosomal protein Heparan-α-glucosamine N-acetyltransferase (HGSNAT). MPS IIIC is part of the lysosomal storage disease (LSD) family and is characterised by the accumulation of glycosaminoglycans (GAGs). This disease has no available treatment and presents early in life with symptoms like missing developmental milestones, neuronal cell loss and loss of motor function, eventually leading to death around the second or third decade of life. Our lab has previously shown that transplantation of hematopoietic stem and progenitor cells (HSPCs) rescue cystinosis, another LSD due to mutations in a transmembrane lysosomal protein. The mechanism of rescue involved lysosomal cross-correction from HSPC-derived macrophages to the disease cells via tunnelling nanotubes within tissues.

We believe that the same principles could be used to treat MPSIIIC. We generated a new MPSIIIC mouse model by knocking-out exon 2 in *Hgsnat*. We have confirmed the presence of MPSIIIC disease phenotypes such as GAG accumulation, enlarged liver, distended bladder, urine retention, lysosomal expansion, neurological defects, increased inflammation, and the presence of disease specific non-reducing end carbohydrates biomarkers in our mouse model. As the first proof of concept, we transplanted *Hgsnat*$^{-/-}$ mice with HSPCs isolated from WT GFP-transgenic mice. Even though we see engraftment of HSPC-derived cells, tissue expression of *Hgsnat* was low and no reduction in GAG storage was observed in the transplanted mice. These data demonstrate that endogenous *Hgsnat* is not well expressed in HSPC-derived macrophages/microglia explaining the limited impact of WT-HSPC transplant on GAGs. Nonetheless, WT-HSPC transplant did show improvement in hepatomegaly, urine retention, neurological defects, and gait defects, which correlates with decreased inflammatory markers in the treated *Hgsnat*$^{-/-}$ mice. Limited efficacy of WT-HPSCs has also been reported in MPSIIIA, which was overcome by using gene-modified HSPCs to overexpress *SGSH* gene. Therefore, we are developing an autologous transplantation of ex-vivo gene modified HSPCs. We have generated self-inactivated (SIN)-lentivirus vector containing human *HGSNAT* cDNA (NM_152419 & XM_005273411.2) and performed *in vitro* testing. Following transduction of mice fibroblasts, human patient fibroblasts and human patient B-lymphoblast, we observed increased human *HGSNAT* mRNA expression, lysosomal localisation of HGSNAT, recovery of HGSNAT enzyme activity, decreased GAG storage, and decreased lysosomal accumulation. We also transplanted gene-modified HSPCs in *Hgsnat*$^{-/-}$ mice, and have confirmed engraftment of our treated mice at 2 months post-transplantation. The results of the *in-vivo* transplants on GAG storage, behavioural outcome, and histological anomalies will be presented at the conference.

650 Enhanced Long-Term Efficacy of an AAV-PAH Vector for Treating Phenylketonuria

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Introduction: Phenylketonuria (PKU), also known as Phenylalanine hydroxylase (PAH) deficiency, is a rare autosomal recessive inherited disorder of phenylalanine (Phe) metabolism, most commonly caused by pathogenetic variants in the *PAH* gene. Hepatic PAH is responsible for the conversion of Phe to tyrosine (Tyr). Deficiency of PAH activity results in hyperphenylalaninaemia (HPA), that is Phe accumulation in the blood and lack of Tyr, and subsequently abnormal brain development in childhood, psychiatric and cognitive problems in adults. The outcomes of current dietary restriction treatment are still suboptimal and pharmacological approaches have drawbacks. The ideal therapy would stably restore PAH activity in the liver and provide a cure for PKU. Several different AAV-mediated *PAH* gene transfer strategies have been developed. However, none of them is successful due to the safety concerns associated with the high doses. **Methods/Results:** By combining an AAV vector with high tropism for liver and an optimized expression cassette, we have developed NGGT002, an rAAV8-based vector expressing human PAH (rAAV8-hPAH). In HepG2 cells with AAV receptor (AAVR) overexpression, a dose-dependent reduction of Phe concentration was observed, indicative of an effective *in vitro* biopotency of hPAH activity of NGGT002. In PKU mouse model maintained on a Phe-containing normal chow diet, complete and sustained correction of HPA phenotypes, such as reduced blood Phe level, increased body weight, darkened coat color and restored brain 5-hydroxyindoleacetic acid (5-HIAA) and Tyr concentration, was observed up to 40 weeks following a single intravenous (IV) injection of NGGT002. Maximal and stable therapeutic effects were achieved at the doses of 1e12 vg/kg in male and 2e12 vg/kg in female PKU mice, much lower than the effective doses reported so far. Robust liver transduction and hPAH expression mediated by NGGT002 were detected in PKU mouse. Furthermore, as assessed by RNAscope in liver samples from single dose NGGT002 IV injected Cynomolgus monkeys on day 56 post administration, *hPAH* expression was detected in approximately 30% of the hepatocytes at the dose of 1.3e13 vg/kg and 50% at 3e13 vg/kg, respectively, demonstrating an effective liver gene transfer and *hPAH* transgene expression in non-human primate (NHP). **Conclusions:** Based on the evaluations in the PKU mouse model, NGGT002 has enhanced effects in correcting HPA phenotypes by effectively expressing hPAH in the liver. In NHP, high level of gene transfer and expression has been achieved. These data suggest that NGGT002 has a substantially improved efficacy and a great potential to safely achieve clinical benefits at a much lower dose for treating PKU patients caused by *PAH* mutations.

651 Silibinin Attenuates Adipose Tissue Inflammation & Reverses Obesity & Its Complications in Diet-Induced Obesity Model in Mice

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Purpose: To investigate therapeutic outcomes of anti-inflammatory activities of the natural compound Silibinin in reversing diet-induced obesity and its complication in mice. Methods: C57BL/6 male mice were fed a high-fat diet for 8 weeks until development of obesity, and then injected with 50 mg/kg silibinin intraperitoneally twice per week, or vehicle for 8 weeks. While on treatment, mice body weight and food intake were monitored, and glucose tolerance test was performed toward the end of the experiment. Animals were sacrified and serum and tissues were collected for biochemical, histological, and gene expression analysis to assess silibinin effects on adipose inflammation, fat accumulation, liver adipogensis and glucose homesatsis. Results: Silibinin reversed adipose tissue inflammation and adipocyte hypertrophy, and blocked diet-induced weight gain and obesity development without affecting food intake. Silibinin also reversed fatty liver disease and restored glucose homeostasis in treated animlas, and reversed hyperglycemia, hyperinsulinemia and hypertriglyceridemia. Conclusion: This study demonstrated that silibinin as an anti-inflammatory therapy is potential alternative to manage obesity and its related pathologies. In addition, silibinin-based therapies could have a promising potential for clinical applications to manage various inflammation-driven disorders.

652 Comparative Analysis of SubQ vs IP Implanted Hepatocyte-Containing Bioprinted Tissues

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Background: Liver transplantation is the standard of care treatment for multiple liver diseases including Acute Liver Failure (ALF) and Urea Cycle Disorders (UCD). While effective, liver transplantation is an invasive surgery with the potential for lifelong complications, including implantation site bleeding, immune suppression, and the potential for new malignancies. As an alternative, some research has been focused on using isolated hepatocytes from livers, unsuitable for transplant, to treat liver diseases. Promising results have been seen in pediatric patients with acute liver failure, where encapsulated hepatocytes are implanted into the intraperitoneal (IP) space to provide a supportive liver function for a limited duration of time. However, it remains to be seen if hepatocyte transplantation can be successful in less invasive surgical sites. The subcutaneous (SubQ) space is comparatively non-invasive, offers larger implant sites, and is less likely to be fouled with the products of liver disease (such as ascites). This study demonstrates that 3D bioprinted tissues containing primary human hepatocytes (PHHs) and mesenchymal stromal cells (MSCs) can be implanted into either the SubQ or IP space of NSG mice with equivalent levels of synthetic function in both implantation sites. **Methods:** Cell spheroids were created by the coaggregation of PHH and MSC cells. Spheroids were then suspended in an alginate-based biomaterial. Aspect Biosystems' microfluidic 3D bioprinter was used to make coaxial 'core-shell' fibre tissues, where a cell-containing core was surrounded by a protective shell material. Liver tissues containing 1 million PHHs and 1 Million MSCs were implanted into the IP or SubQ space of immunodeficient NSG mice. Blood was collected one week prior to implantation and at regular intervals post-implantation. Implanted liver tissue function was assessed using human albumin quantified via ELISA over a period of 35 days. **Results:** Prior to tissue implantation, circulating human albumin levels in NSG mice were below the level of detection. Mice receiving bioprinted liver tissues into the IP space demonstrated plasma human albumin levels of 1137 ng/mL one day after implantation. These levels rose to 1970 ng/mL by week two and subsequently dropped to 1176 ng/mL on week 5. Mice receiving bioprinted liver tissues in the SubQ space had plasma human albumin levels reaching 793 ng/mL by day 1, 1970 ng/mL on week 2, and 2031 ng/mL on week 5. Statistical analysis did not show a significant difference between implantation sites. Mice receiving fibres implanted into the SubQ space had faster closure of the surgical wound and decreased bruising compared to mice with fibres implanted into the IP space. **Conclusion:** 3D bioprinted implantable liver tissues maintained high levels of synthetic function after implantation into the IP or SubQ space of NSG mice over a period of a month. The equivalency in function between implantation sites suggests that minimally invasive surgical options may have several benefits. The SubQ space has the potential to receive larger therapeutic tissues, with more frequent replacement and easier monitoring. The decreased invasiveness of the procedure can make liver cell therapies an attractive option for other non-life-threatening illnesses. Our next goals are to confirm that bioprinted liver tissues implanted SubQ can effectively treat a mouse model of metabolic liver disease, and to examine the function of larger bioprinted tissues within the SubQ space of rats.

653 Liver Directed Lentiviral Gene Therapy Provides Long Term Phenotypic Correction of Methylmalonic Acidemia in a Mouse Model

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Methylmalonic acidemia (MMA) is a severe inborn error of metabolism caused by deficiency of methylmalonil-coA mutase (MUT), leading to accumulation of the toxic methylmalonic acid. The significant MMA-related morbidity and mortality in infancy led to perform liver transplantation as elective treatment, and to explore gene therapy in MMA mouse models as an alternative therapeutic option. We exploited lentiviral vector (LV) mediated liver gene therapy, which would potentially allow for a stable gene transfer even in paediatric patients at the first disease stages by virtue of LV genomic integration. We generated and validated LV encoding a human MUT transgene under the control of a hepatocyte-specific expression cassette (LV.MUT). We assessed LV-mediated liver gene therapy in a mouse model of MMA expressing a compensatory MUT transgene in the muscles (MCK-Mut-/-). We intravenously administered 2-week old MCK-Mut-/- mice (n=8) with LV.MUT at a dose of $5x10^{10}$ transducing units (TU)/kg and monitored them for >1 year. The gene therapy resulted in a complete rescue of the survival rate and the growth curve of MCK-Mut-/- animals, comparable to that of wild-type (WT) controls. We observed a rapid and sustained decrease of circulating methylmalonic acid (cMMA; mean of 146 μM and 979 μM in treated vs. untreated animals 12 months post-LV) and a substantial 60-fold reduction in the intrahepatic concentration of methylmalonic acid in treated mice compared to MCK-Mut-/- controls. We achieved efficient LV-mediated liver gene transfer with ~15-20% transduced liver area, as assessed by RNA in situ hybridization (ISH). We observed liver overexpression of MUT in MCK-Mut-/- transduced mice compared to endogenous MUT in the liver of WT mice, as assessed by mRNA and protein analyses. End-point histopathology analysis showed that livers of MCK-Mut-/- untreated mice present with enlargement of hepatocyte with small eosinophilic droplets and autophagic vacuoles, which were not observed in age-matched WT controls or MCK-Mut-/- treated with LV. These data suggest a possible detoxification activity of the corrected hepatocytes over the non-corrected ones. Since MCK-Mut-/- mice display hepatorenal mitochondrial pathology similar to MMA patients, we performed electron microscopy on liver and kidney samples from treated mice, showing rescue of mitochondriopathy in both hepatocytes and epithelial cells of the proximal tubules (median mitochondrial area and shape comparable to that of WT mice). These results correlated with normalization of plasma FGF-21, a key biomarker of the mitochondrial function which is highly dysregulated in MMA (mean of 176 and 2362 pg/ml in treated vs. untreated animals

12 months post-LV). We treated additional MCK-Mut mice at different LV doses (2.5, 5 or 10x10[10] TU/kg) and observed weight normalization at all doses and a dose-dependent reduction of cMMA, which reached a plateau at ~180 μM between the mid and high dose. These data provide evidence for long-lasting therapeutic benefit in MMA mouse model with LV gene therapy.

654 Improving *Ex Vivo* HSPCs Strategy for Pompe Disease: CX3CR1 Intron 4 Targeting as Safe Harbour and Novel Mice Models for Its Preclinical Study

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Ex-vivo gene therapy success exploiting Haematopoietic Stem Progenitor Cells (HSPCs) for paracrine cross-correction is undeniable, like in the case of the rare pathology of Pompe Disease (PD) using lentiviral vectors. However, the semi-random integrations of these vectors rise many safety concerns. Although genome editing (GE) stands as a safer alternative since we can select the insertion site of the expression cassette; before its translation into clinic there are several drawbacks that need improvements, such as safety, efficient expression of the therapeutic transgene and stem cell repopulation. Furthermore, suitable animal models are required to asses safety and therapeutic efficacy before its translation into clinic. Our GE strategy intends to obtain high levels of the transgene expression on the myeloid linage after HSPCs GE. We successfully targeted the 4th intron of CX3CR1 with CRISPR-Cas9 on HSPCs without phenotypic or locus expression alteration, acting as a safe harbour. We designed different AAV6 donor DNA templates including a spleen-focus-forming-virus (SFFV) promoter expressing eGFP as reporter. Unexpectedly, when specific targeted insertion was achieved, we observed increased levels of CX3CR1 protein and mRNA on HSPCs, but not in the colonies from CFU's assays. Withal, neither edited T cells nor THP1 cells showed any CX3CR1 increment after targeted addition. Furthermore, we documented a myeloid skewed expression pattern of our reporter transgene. Our observations suggest that this CX3CR1 increment is restricted to discrete HSPCs stem sub-populations. Interestingly, the increase of CX3CR1 could facilitate chemotaxis of these cells towards inflamed tissues. Preclinical tests on NSG humanized mice were made, and no genotoxic or deleterious effects were identified when using our reporter transgene. Therefore, encouraging the use of CX3CR1 as a safe harbour for targeted insertion on HSPCs. However, future testing of the therapeutic GE strategies for PD present several limitations due to the current PD mice models. As these mice models are immunocompetent, show a mixture phenotype of adult and non-classic infantile onset, lack early mortality and present slower muscle weakness. To address these bottlenecks, we have just engineer two novel PD mouse models using CRISPR-Cas9, which mimic two common PD mutations described on patients: c.525del and c.483dup.

655 Long-Term Effects of Fat-Directed FGF21 Gene Therapy in Aged Female Mice

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Adipose tissue plays a pivotal role in energy homeostasis and pathologies of metabolic diseases. Aging is associated with an increase in adiposity and a functional decline in muscle. Hormone fibroblast growth factor 21 (FGF21) is increasingly recognized as a promising therapeutic agent for metabolic diseases. Rec2, a novel engineered hybrid serotype, has recently emerged as a main choice of AAV serotypes for targeting adipose tissue in vivo. However, Rec2 also transduces liver. The off-target effects in liver are becoming a challenging issue in AAV application. To achieve lower and safer vector dose, we recently designed a single AAV cis-plasmid harboring dual expression cassettes: one using CBA promoter to drive transgene and another using liver-specific albumin promoter to drive a microRNA targeting WPRE sequence in this cis-plasmid. When packed to Rec2, this dual-cassette vector transduces adipose tissue in a highly selective manner, while significantly restricting off-target transduction of liver. With this fat directed Rec2 dual-cassette vector, we have recently demonstrated that FGF21 gene transfer improved metabolic and immune health in insulin resistant BTBR mouse model. FGF21 has also gained an attention as a longevity associated gene. In this study, we deliver Rec2 dual cassette vector to visceral fat of 17-month-old female mice to investigate 1) can FGF21gene transfer in adipose tissue produce high and sustainable level of serum FGF21, 2) can long term FGF21 gene transfer improve aging-related functional decline. Animals with FGF21 gene delivery displayed a steady, significant lower body weight over 7-month course of the study compared to age-matched control mice. The serum level of FGF21 in the gene delivery mice was almost 100 folds more than that in the control mice. No toxicity was observed in FGF21 gene delivery animal. FGF21 gene delivery reduced adiposity and increased relative lean mass and energy expenditure. However, those changes were not translated into benefits on functional improvement of muscle and liver. Overall, we have demonstrated that a single dose of fat-directed AAV-FGF21 gene delivery can provide a sustainable, high serum level of FGF21 with safe profiles over long time of the period, and mostly influences adipose tissue homeostasis and energy expenditure. High levels of FGF21 alone in aged mice is not sufficient to facilitate liver or muscle functions. **CONFLICT OF INTEREST** L.C. and W.H. are inventors of a patent application related to the liver-restricting AAV vector. L.C. is co-founder of Zvelt Therapeutics. All other authors declare no conflicts of interest.

656 Development of Gene Therapy Vectors for the Treatment of Maple Syrup Urine Disease

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There is a significant clinical need for novel therapies targeting inborn errors of metabolism. Perinatal administration could provide early mitigation of developmental complications and enhanced biodistribution to target organs. We are investigating the feasibility of a gene therapy strategy to treat maple syrup urine disease (MSUD), a branched chain amino acids (BCAA) metabolic disorder that is caused by mutations in the branched chain ketoacid dehydrogenase (BCKDH) complex. MSUD is associated with neurological complications, psychiatric manifestations and early death. Current treatments are limited to BCAA dietary restriction, blood transfusion and liver transplantation. We have utilized an MSUD mouse model wherein the mouse *Dbt* (*mDBT*) gene, which encodes a constituent subunit of the BCKDH complex, has been knocked out. We compared adeno-associated virus (AAV) and lentiviral (LV) vectors, administering them once intravenously to newborn homozygous knockout mice. We tested two AAV9 vectors, which encoded the codon-optimized human *DBT* (*hDBT*) coding sequence (CDS) under the control of either a ubiquitous (1.3E11 vg/mouse; n=5) or a liver-specific (1.3E11 vg/mouse; n=9) promoter. We also tested two LVs, containing the liver-specific promoter and codon-optimized versions of either *mDBT* (9E7 TU/mouse; n=7) or *hDBT* (7E7 TU/mouse; n=7) CDS. Treated and control mice were followed for five weeks post-injection, and tissues were collected for vector copy number and mRNA expression analyses. All vectors resulted in significant enhancements in survival of treated mice. The AAVs resulted in more widespread expression across different organs than LVs. The LV encoding *mDBT* achieved the greatest level of hepatic expression. The *hDBT* LV and liver-specific AAV9 resulted in comparable hepatic expression, which was significantly greater than the wildtype *mDBT* level. The ubiquitous AAV9 vector resulted in the lowest hepatic expression among the vectors tested. In conclusion, this study shows that AAVs or LVs have potential application as hepatic gene therapy platforms in this mouse model. Future work will include toxicity studies, as well as comprehensive characterization of metabolic and behavioral correction over a prolonged survival course.

657 Improvement of Progressive Familiar Intrahepatic Cholestasis Type 2 via AAV-Mediated Hepatic BSEP Expression in Mice

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is a rare autosomal recessive disorder caused by mutations in *ABCB11* gene encoding for the liver bile salt export pump (BSEP), responsible for the transport of bile salts (BS) from hepatocytes to the canalicular lumen. BSEP absence or dysfunction results in impaired BS secretion and its accumulation in hepatocytes leading to severe liver damage. PFIC2 patients usually develop symptoms during infancy, including cholestasis, failure to thrive, jaundice, hepatomegaly and severe pruritus. Most develop early fibrosis and end-stage liver disease and have an increased risk of developing hepatocellular carcinoma. Therapeutic approaches include pharmacological management of symptoms, surgical biliary diversion or ultimately liver transplantation, which currently is the only curative option. Adeno associated virus (AAV)-based gene therapy targeting the liver represents a safe and efficient option for PFIC2 patients by restoring long-term hepatic BSEP expression and physiological bile secretion. We first generated and characterized an *Abcb11*$^{-/-}$ mouse model of PFIC2 in a pure C57BL/6 genetic background. Bile salt secretion was dramatically impaired in both sexes with reduced BS levels in the bile and small intestine. Female mice showed a progressive elevation of serum transaminase and bilirubin levels starting at two months of age, while male mice only showed an increase in these biomarkers after six months of age. Hepatomegaly was observed in both genders but was significantly higher in females. Overall, females presented a more severe phenotype and recapitulated the human disease more closely than males. We next assessed the therapeutic efficacy of gene therapy in this PFIC2 mouse model using a liver-tropic AAV8 vector carrying a codon-optimized human BSEP cDNA under the control of a liver-specific promoter (VTX-802(8)). Five-week-old PFIC2 female mice were injected with a single intravenous administration of VTX-802(8) at two different doses and followed until four months of age. Correct localization of BSEP in biliary canaliculi was observed in treated mice after sacrifice at either dose. Animals treated with the higher dose showed normalization of serum transaminase and bilirubin levels at three weeks post-injection. Moreover, these mice showed a partial but significant reversion of hepatomegaly, and the release of BS from the liver to bile and small intestine was significantly increased at 4- and 11-weeks post-injection, indicating that physiological bile secretion was partially restored. These early results indicate that VTX-802(8) has therapeutic potential for PFIC2. However, further development of the AAV vector and/or combination with other pharmacological products, might be investigated to enhance treatment efficacy and further revert the disease biomarkers. The development of a durable cure for PFIC2 and its translation to the clinic would provide a life-changing alternative for these pediatric patients with high unmet medical need.

658 Combination of CD4+ and CD8+ in HBV T-cell Therapy Enhances Virus Control by Increasing IFN-γ and TNF-α Secretion

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As most therapies so far focus on $CD8^+$ cells, the role of $CD4^+$ T cell help in T-cell therapy has merely been investigated. T-cell based therapy is a promising means to treat chronic hepatitis B virus (HBV) infection and HBV-associated hepatocellular carcinoma. T cells engineered to express an HBV-specific T cell receptor (TCR) may cure an HBV infection upon adoptive transfer. We here investigate whether the addition of redirected $CD4^+$ T cells supports $CD8^+$ TCR-T cells. Recently generated MHC class I as well as MHC class II (MHC II)-restricted TCRs allowed to redirect T cells and eliminate HBV in cell culture. Both, $CD8^+$ and $CD4^+$ T cells, engrafted with HBV-specific TCRs secreted IFN-γ and TNF-α and killed HBV-positive target cells. Furthermore, the transfer of MHC I-restricted TCRs into a humanized mouse model infected with HBV caused a significant decline of virological markers with limited injury of the liver. In a next step, we investigated the therapeutic potential of MHC II-restricted HBV core-specific $CD4^+$ T cells and MHC I-restricted HBV surface-specific $CD8^+$ T cells alone and in combination in AAV-HBV-infected mice, modeling a chronic HBV infection upon restimulation with peptides Even if all groups could control HBV infection by lowering HBeAg and HBsAg, in the combination group, virological markers declined more pronounced by 2 to 3 logs. Better clearing of HBV antigens was accompanied by increased IFN-γ and TNF-α secretion by $CD4^+$ and $CD8^+$ T cells from the liver and spleen. Despite higher cytokine levels, the liver damage was kept to a minimum with alanine aminotransferase levels peaking between days 15 and 22 and normalizing again thereafter. Based on these data, we hypothesize a positive helper effect of $CD4^+$ T cells by stimulating $CD8^+$ cells and endogenous cytokine secretion.

659 *In Vivo* Screening Towards Hepatic Stellate Cell Targeting in Non-Alcoholic Steatohepatitis

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Non-alcoholic steatohepatitis (NASH) is a liver disease characterized by liver inflammation, fat buildup, and fibrosis modulated by a wide population of liver cell types such as hepatocytes and hepatic stellate cells. To effectively provide treatment to patients, it is important to specifically target cell-types with different interventions and tightly modulate expression of the transgene with a modality such as AAVs. Current AAV screening methods are unable to capture transgene expression at the single-cell level. Using single-cell RNA sequencing with expressed barcodes, we have developed a platform to give us transduction information in specific cell-types. Using this screening platform, we have been able to identify some cell-type specific AAV serotypes for liver-targeting and run head-to-head comparisons of regulatory elements to identify the best promoters for expression. We injected a pool of 17 serotypes, at a dose of 1E11 per serotype, into 3 mouse models to study fibrotic liver disease (n = 3 per model): carbon tetrachloride (CCl4), trans-fat containing amylin (AMLN) liver diet, and a non-diseased control. 2 and 4 weeks after AAV injection, we isolated and enriched for populations of transduced stellate cells and hepatocytes. These cell populations were sorted and ran through the 10x Genomics single-cell system. Subsequently RNA was sequenced using next-generation sequencing, and data was analyzed to find AAV serotypes that transduce specific cell-types. We find that several AAVs already validated in literature such as AAVrh10, AAVDJ8, AAVDJ9, and AAVDJ had high transduction in hepatocytes, higher than AAV8 that is actively used in liver clinical trials. From those screening results, we were able to identify several novel serotypes that are present in cholangiocytes and hepatic stellate cells. This screening platform was applied to 47 AAV transgene constructs of regulatory elements to increase transduction in hepatocytes in non-diseased and AMLN mice. Using this technology, we were able to identify a promoter in hepatocytes that had 2x RNA transcripts per cell higher than the albumin promoter. Using our screening platform, we were able to identify AAV serotypes with cell-type specificity and also measure their expression levels in disease relevant contexts. With this information, we have used our optimized AAV vector to screen therapeutic constructs in mice as well as large animal models, such as non-human primates.

661 Pigment Epithelium-Derived Factor (PEDF) Short Peptides Enhance the Proliferation of Articular Chondrocytes in Cell Culture, Induce Chondrocyte Trans-Differentiation of Mesenchymal Stem Cells and Promote the Regeneration of Damaged Cartilage in an Animal Model of Osteoarthritis

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Pigment epithelium-derived factor (PEDF) is shown to promote the expansion of corneal limbal stem cell and liver stem cell in cell culture. This make PEDF a potential tool in cell therapy. Here we report the effect of a PEDF short peptide (PSP) on the expansion of articular chondrocytes in cell culture. Primary chondrocytes were isolated from rat knee and cultivated in the presence of the PSP for 12 days. In this condition, chondrocytes not only grew faster, but also aggregated into huge three-dimensional colonies. In these colonies, PSP treatment markedly increased the numbers of aggrecan-positive cells, indicating the identity of chondrocytes. Moreover, PSP can promote chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs), as evident by the increased expression of chondrogenic marker genes and proteoglycans, as well as the phosphorylation of STAT3. Pharmacological inhibitors of PEDF receptor and STAT3 signaling dramatically blocked the effect of PSP on cultured MSCs. In addition, we investigated whether the PSP is able to induce cartilage regeneration in rat knees in which cartilage is already destroyed by monoacetate injection for 7 days. PSP was injected into knee joints twice, at 4-day intervals. When joint tissues were examined 10 days later, PSP induced extensive chondrocyte regeneration in the damaged cartilage, demonstrated by double immunostaining of BrdU to

track DNA synthesis and chondrocyte markers, including aggrecan, collagen type 2 and Sox9. In conclusion, PSP has the ability to promote cartilage regeneration, possibility through the expansion of remaining chondrocytes or the induction of trans-differentiation of BMSCs.

662 AUF1 Gene Therapy for Traumatic Skeletal Muscle Injury- Significantly Accelerated Muscle Regeneration in Preclinical Animal Models

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Traumatic skeletal muscle injuries are the most common debilitating injuries, whether in military service, sports, or accidents in everyday life. Traumatic muscle injury can become muscle wasting disease, with unproductive cycles of attempted muscle regeneration, exhaustion of muscle stem (satellite) cells, and continued degeneration and atrophy of the wounded muscle. Surgical repair of traumatic muscle injuries typically does little to improve muscle regeneration and quality of life. There are currently no FDA approved therapeutic interventions to promote muscle regeneration following traumatic muscle injury. There is, therefore, a large unmet need to develop therapeutic, approaches that significantly activate and restore satellite cells, promote successful muscle regeneration, reduce muscle atrophy and necrosis, and reduce the extensive time for muscle regeneration following traumatic injury. Following injury, muscle healing occurs in 3 phases over a period of 1 month in mice and 3-4 months in human: (1) Degeneration/necrosis, inflammation followed by its cessation; (2) Muscle regeneration, repair; and (3) Maturation and remodeling of muscle fibers (myofibers). All three processes, inhibition of inflammation and necrosis, regeneration and maturation of muscle need to be stimulated, which has not been currently achievable. AU-rich mRNA binding factor 1 (AUF1) is an RNA binding protein that binds repeated AU-rich elements (AREs) located in the 3' untranslated region of approximately 3% of mRNAs. ARE targets mRNAs for either rapid degradation, stabilization and/or increased translation, largely controlled by AUF1. We previously showed that in skeletal muscle, the majority of key regulators of satellite cell activation and maintenance, muscle regeneration, and mitochondrial biogenesis essential for muscle function, are encoded by ARE-mRNAs regulated by AUF1. Here, in preclinical animal models, we used supplementation of muscle AUF1 delivered to satellite and muscle cells by either systemic muscle specific AAV8-tMCK-AUF1 or lentivirus vector-AUF1 direct muscle administration gene therapy either during or prior muscle injury. We demonstrate following BaCl2 induced traumatic tibialis anterior (TA) muscle injury, that AUF1 gene therapy: (1) Reduces TA muscle atrophy, (2) Results in more rapid expression of satellite cell activation and myoblast differentiation markers including Pax7, MyoD and Myf5, while atrophic gene expression is strongly reduced as early as 3 days post-injury; (3) Increases expression of myozenins, indicators of myofiber maturity and muscle regeneration progression; (4) Rapidly reduces the expression of embryonic myosin Myh3, a marker of productive myogenesis; (5) Enhances the size of differentiated mature neo-synthesized myofibers, while reducing their percent, observable as early as 5-7 days post-injury; and (6) Dramatically reduces the time of post-injury muscle regeneration, including time for restoration of normal muscle histological morphology. Finally, genome-wide transcriptome and translatome analysis of C2C12 myoblasts cells expressing AUF1 from a lentivirus vector confirmed the pro-myogenic function of AUF1, which increases protein synthesis, induced upregulation of mRNAs that encode muscle contraction and regeneration pathways, and downregulation of mRNAs that encode inflammatory factors. These studies suggest that AUF1 overexpression accelerates muscle regeneration following traumatic injury and should be evaluated further as a candidate for human gene therapy.

663 Assessment of Therapeutical Potential and Advantage of a Dual AAV Approach for Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is a severe rare pediatric disease that affects skeletal and cardiac muscles, leading to progressive muscle wasting and premature death. DMD is caused by X-linked mutations in the dystrophin gene that result in lack of dystrophin, a crucial protein required for the biomechanical support of muscle fibers and, as recently identified, for signaling function. Although DMD is a suitable disease for gene replacement therapy, the extent of Dystrophin transcript size exceeds by far the AAVs cargo capacity and only short versions of dystrophin (μDys) can be accommodated. Several forms of μDys are currently being investigated in clinical trials. However, while the therapeutic effects are unequivocally met in the animal models, results from clinical trials highlighted an insufficient correction of the disease and raised safety concerns due to the appearance of severe adverse events (AE) triggered by immunogenic reactions after vector delivery. This evidence supports the necessity to the identify strategies to face the challenges of safety and efficacy. It is still unclear to which extent microdystrophins can really replace the full-length dystrophin function in human patients. Our strategy is to generate a larger version of dystrophin (quasidystrophin-qDys EP2020/05837) including key additional structural and functional domains by a dual system based on homologous recombination of the overlapping region of two AAVs vectors. We first checked efficiency and safety parameters of our system by employing an innovative ddPCR-based methodology by which we proved that our Dual AAV approach, systemically administered in DBA2mdx mice, led to high transgene reconstitution efficiency and negligible ITRs-dependent concatemerization, and consequently to remarkable protein restoration in muscles. Our data indicated that mice treated systemically with Dual AAV-qDys displayed global improvement of muscle pathology and function. In particular, DBA2 mdx mice treated with qDys have lower fibrosis and higher muscle force and grip strength as compared to mice expressing similar amount of microdystrophin protein. The histological and functional amelioration was also associated with increased stability of the dystrophin-associated complex. Of note, no adverse effects were observed in these experiments. In conclusion, the efficiency of the system, by allowing the use of a safe dosing, supported the suitability of qDys for therapeutic investigation. The more performant gene candidate qDys together with the use of a new AAV capsid allowing a lowering of dose and preventing liver toxicity, has the potential to increase the correction of

the disease at higher level than the current uDys approaches, keeping in mind that supportive strategies might be needed to further improve first-generation gene therapies.

664 Preclinical Development of MyoDys45-55, a Gene Editing Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy is a fatal muscle wasting disease typically caused by out-of-frame mutations in the *DMD* gene. Some therapeutic approaches for Duchenne aim to restore the reading frame and produce an internally deleted dystrophin protein similar to what occurs in the milder Becker muscular dystrophy. One way to achieve this reframing is with CRISPR/Cas9 gene editing. Gene editing offers the advantage of permanently changing the patient's own DNA and being able to retain more of the protein coding sequence than AAV-based gene replacement therapies. We have developed a gene editing therapy, MyoDys45-55, that generates an in-frame deletion of exons 45-55 and encompasses a hotspot of 50% of Duchenne patient mutations. We have demonstrated AAV delivery of MyoDys45-55 can restore dystrophin and function in a humanized mouse model of Duchenne. We have also tested this strategy in combination with immune suppression to dampen the immune response to AAV and Cas9. Here we describe preclinical studies assessing the efficacy, biodistribution, safety, and immune response to AAV-MyoDys45-55 in vivo. This work will advance development of this potential therapeutic for Duchenne that can permanently restore the reading frame for a large proportion of patients.

665 Smoking and Tetramer Tryptase Accelerate Intervertebral Disc Degeneration by Inducing METTL14-Mediated DIXDC1 m6 Modification

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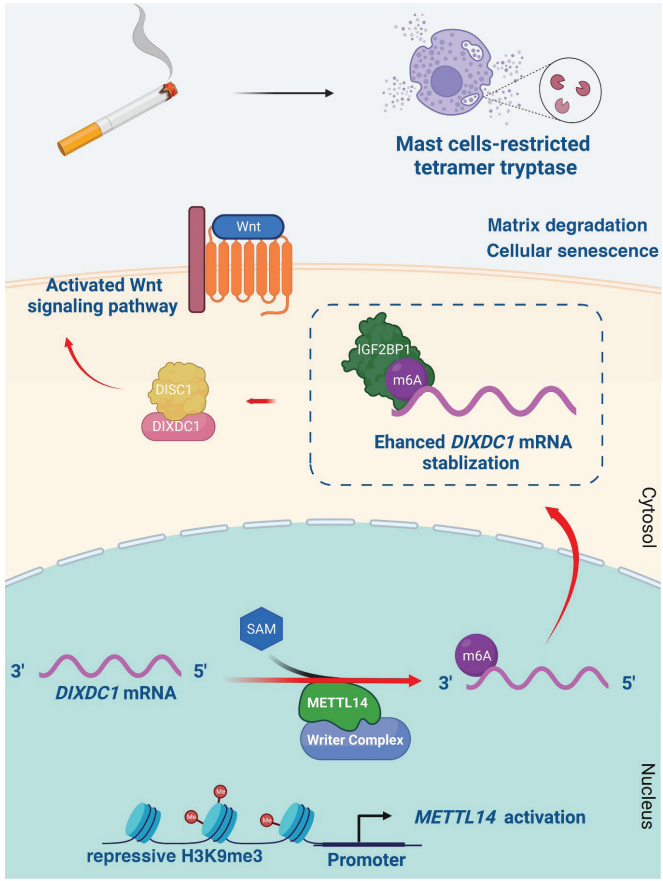
Although cigarette smoke (CS) and low back pain (LBP) are common worldwide, their correlations and the mechanisms of action remain unclear. We had shown that excessive activation of Mast Cells (MCs) and their proteases play key roles in CS associated diseases, like asthma, chronic obstructive pulmonary disease (COPD), blood coagulation and lung cancer. Previous studies also show that MCs and their proteases induce degenerative musculoskeletal disease. By using mice custom-designed smoke-exposure system, we demonstrated that CS results in intervertebral disc (IVDs) degeneration and the release of MC-restricted tetramer tryptases (TT) inside the IVDs. TT was found to regulate the expression of methyltransferase 14 (METTL14) at the epigenetic level by inducing N6-methyladenosine (m6A) deposition in the 3'-untranslated region (3'-UTR) of the transcript that encodes DIX Domain Containing 1 (*DIXDC1*). That reaction increases the mRNA stability and expression of *Dixdc1*. DIXDC1 functionally interacts with 'Disrupted in Schizophrenia-1' (DISC1) to accelerate the degeneration and senescence of nucleus pulposus (NP) cells by activating a canonical Wnt pathway. Our study demonstrates the association between CS, MC-derived TTs, and LBP. These findings raise the possibility that METTL14-medicated DIXDC1 m6A modification could serve as a potential therapeutic target to block the development of degeneration of the NP in LBP patients.



666 AAV.U7snRNA as a Platform to Deliver Antisense Sequences for mRNA Splicing Modification to Treat Neuromuscular Disorders

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Vectorized Exon skipping (VES) is a promising approach to treat neuromuscular disorders (e.g. Duchenne muscular dystrophy (DMD), Myotonic Dystrophy type (DM1)). It is based on restoring the reading frame of mRNA or interfere with toxic transcripts. Conventional approaches using antisense oligonucleotides (ASO) have several limitations such as the need of repeated injections due to their lack of stability and their limited tissue tropism into affected organs (e.g., heart and others). VES constructs packaged into AAV vectors are

able to overcome these limitations as the ASO is embedded into the sequence of a small nuclear RNA with its own promoter, thereby taking advantage of the continuous expression of AAV transgene cassettes and the better tissue access of AAVs over ASOs. This approach is currently being tested in a clinical trial for *DMD* exon 2 duplication (NCT04240314) and has been proven to be safe and well tolerated. Importantly, efficient exon skipping in muscle and increased levels of dystrophin protein were observed in all three treated patients. Based on these exciting results, we have developed additional VES vectors for new regions of the DMD gene: exon 44 and exons 6-8 multi-exon skipping. For the exon 44 program, several VES were tested *in vitro* in patient derived cells harboring different mutation surrounding or within exon 44. We also tested the most promising VES *in vivo* 3 months post intramuscular injection (IM) in a humanized mouse model carrying an exon 45 deletion (hDMD del45). In both models, around 85% of exon skipping was achieved, resulting in around 90% of truncated dystrophin expression. In hDMD del45, more than 50% force improvement and a significant reduction of inflammation were observed in the treated muscle. Minimal efficacious dose studies were also performed to determine the best AAV dose following systemic delivery. 3-month post-injection, similar results were obtained: efficient skipping, protein restoration and force improvement. We performed the same approach for exons 6-8 since we know based on literature that in canine models of DMD, skipping of exons 6-8 (CH2 domain) produce a highly functional dystrophin. Thus, skipping of exons 6-8 could be a promising treatment option for 4% of DMD patients. Exon skipping and dystrophin expression were evaluated *in vitro* using patient cell line harboring mutation with exons 6-8 and in our new humanized mouse model that contains a nonsense mutation in exon 7 (hDMD ex7ns). Both *in vitro* and *in vivo* data 3-months post IM data demonstrated that one of our VES could induced efficient exon skipping, resulting in around 50-70% of truncated dystrophin expression and significant increase in muscle eccentric force. To conclude, our lead candidates induce efficient *DMD* exon 44 or exons 6-8 skipping, resulting into dystrophin production and muscle strength improvement in major muscle groups affected in DMD. These two new AAV.U7snRNA skipping vectors represent promising gene therapy candidates for ~6-12% of DMD patients and will allow the production of nearly full-length, an advantage over micro-dystrophin approaches.

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667 Precise Correction of Duchenne Muscular Dystrophy with Exon 51 Deletion by Adenine Base Editing-Induced Exon Skipping in a Humanized Mouse Model

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Background: Duchenne muscular dystrophy (DMD) is the most common lethal, X-linked genetic muscle disease, caused by mutations in the *DMD* gene. While there are thousands of documented clinical mutations, nearly 60% of DMD-causing mutations occur in a "hotspot" region encompassing exons 43 to 55 of the *DMD* gene that encodes the central rod domain of dystrophin protein. Here, we target the correction of the *DMD* exon51 deletion mutation by exon-skipping therapy of the exon50 which may therapeutically benefit DMD patients. **Methods:** We generated a genetically humanized DMD murine model by replacing the mouse exon50 with human exon50, followed by the deletion of mouse exon51 ($DMD^{\Delta mE50,51,\ hE50KI}$), which disrupted the dystrophin open reading frames. Then, we explored the potential of using adenine base editor (ABE) to modify the splice donor sites of the *DMD* gene, causing skipping of a common *DMD* deletion mutation of exon51 by intramuscular injection and systemic delivery of AAV vectors. Then, we evaluated the extent of exon skipping, dystrophin restoration, and phenotypic improvements of cardiac and skeletal muscles 6 weeks after the treatment. **Results:** Humanized $DMD^{\Delta mE50,51,\ hE50KI}$ mice recapitulated many aspects of DMD pathological characterization, including the absence of dystrophin, inflammatory infiltration, muscle weakness, and creatine kinase (CK) release. Intramuscular injection of $DMD^{\Delta mE50,51,\ hE50KI}$ mice with AAV encoding ABE components as a split-intein trans-splicing system instituted >90% of the targeted exon-skipping in the DMD transcripts and restored up to 60% dystrophin *in vivo*. Furthermore, systemic delivery also efficiently restored dystrophin expression and ameliorated pathologic hallmarks of DMD, including histopathology, serum CK level and grip strength (Fig. 1). **Conclusions:** This unique DMD mouse model with the human genomic sequence allows *in vivo* assessment of clinically relevant CRISPR ABE gene-editing strategies as well as other therapeutic approaches and represents a significant step toward therapeutic translation of adenine base editing for correction of patients with DMD or other genetic neuromuscular diseases.

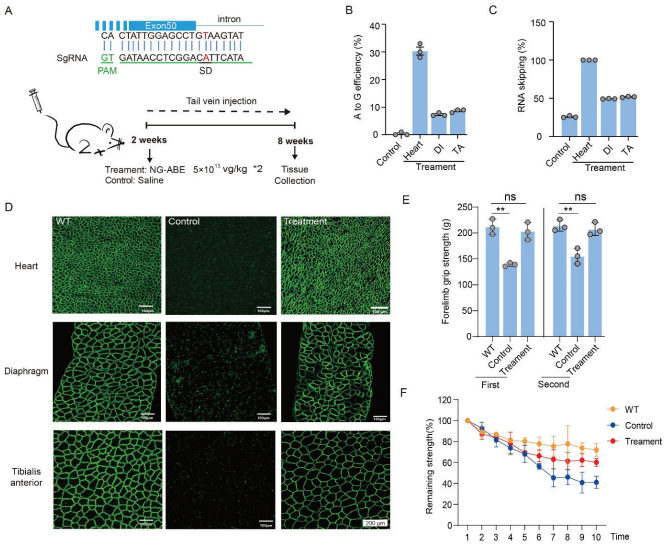

Figure 1. Intravenous delivery of NG-ABE system efficiently rescues dystrophin expression and muscle function in humanized DMD mice. A, Schematic of intravenous administration of NG-ABE particles and illustration of the human exon50 sgRNA binding position in SD. Sequence shows sgRNA (green line), target adenine (red) and PAM (green). Black arrows indicate time points for tissue collection after tail vein injection. Measurement by deep sequencing of splicing site mutation (B) and exon skipping (C) in tibialis anterior, diaphragm and heart after systemic delivery (n=3). D, Immunohistochemistry analysis shows restoration of dystrophin expression in the multi-muscle of DMD$^{\Delta hE50,h51,hE50hG}$ mice 6 weeks after injection. Dystrophin was shown in green. Scale bar, 200 μm. Forelimb grip strength (E) and remaining strength (F) were measured in WT, DMD$^{\Delta hE50,h51,hE50hG}$ mice, and DMD $^{\Delta hE50,h51,hE50hG}$ mice treated with NG-ABE particles (n=3). Data shown as mean ± SEM. Different asterisks represent statistical significance (P<0.01) in multiple comparison test using ANOVA.

668 Addition of a Protein Domain from the Dystrophin C-Terminus Improves Functional and Biochemical Properties of AAV-Encoded Microdystrophin

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Duchenne Muscular Dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes dystrophin, an essential component of the dystrophin-associated protein complex (DAPC) that forms a critical link between intracellular actin and extracellular laminin. The coding sequence for dystrophin is significantly larger than the packaging capacity for AAV, which has led to the development of engineered "microdystrophins" (μDys). These miniaturized dystrophin proteins rely on the modular structure of dystrophin, which contains several structurally homologous sections termed "spectrin-like repeats". Genotype analysis from patients with Becker Muscular Dystrophy, a milder allelic variant of DMD, indicates deletion of a number of these repeats may have a minimal impact on the protein function. However, the precise composition of dystrophin elements that should be retained within μDys to confer optimized functionality is still a matter of debate. The dystrophin C-Terminal (CT) region, which comprises 325 amino acids from the critical cysteine-rich domain to the carboxy-terminus, is a prominent example of this. Evidence suggests that the dystrophin CT region targets several proteins to the sarcolemma, including the signaling adapter syntrophin, and dystrobrevin which reportedly stabilizes DAPC in cardiac and skeletal muscle. This distal CT region has also been implicated in actin binding and dynamics with the DAPC. Early work with intramuscular administration of AAV.μDys showed improved muscle force with a μDys containing a longer CT region in *mdx* mice, a preclinical model of DMD. We have recently reported that systemically administered AAV.μDys with a longer CT region can facilitate recruitment of syntrophin and nNOS and may increase μDys protein stability; however, a functional comparison between μDys proteins with/without an extended CT region is lacking. To directly test whether the CT region contributes to μDys functionality we produced two vectors encoding versions of μDys under a muscle specific promoter without ("μDysCT48") or with ("μDysCT194") helix 1 of the coiled coil motif that includes binding sites for syntrophin and dystrobrevin. Vectors were administered via single intravenous injection into the tail vein at a dose of $5x10^{13}$ GC/kg in male *mdx* mice at 5 weeks of age. 12 weeks after dosing, forelimb muscle function was assayed by grip strength, followed by *in vitro* force measurements at necropsy. Both μDys increased body weight-normalized grip strength to near wild-type (WT) levels (μDysCT48 = 96.1%, μDysCT194 = 96.31%, compared to 85.83% for *mdx*). To more sensitively measure and compare muscle force effects produced by the two μDys, extensor digitorum longus (EDL) muscles were excised and subjected to *in vitro* force measurement followed by a protocol of repeated eccentric contractions. The μDysCT48 increased EDL specific force by 24.69% relative to *mdx* (to 62.83% of WT). In contrast, μDysCT194 led to significantly higher EDL force than μDysCT48 (51.92% increase over *mdx* μDysCT48, to 76.56% of WT). After 5 eccentric contractions, μDysCT194-treated EDLs produced 84.69% of initial force, which was significantly increased over *mdx* (78.21%), while μDysCT48-treated EDLs produced 81.62% of initial force (not significant vs. *mdx*). Altogether, our results suggest that an extended dystrophin CT region optimizes μDys functionality, thus warranting further development of μDysCT194 as a promising gene therapy candidate for DMD.

669 An AAV-shRNA DUX4-Based Therapy to Treat Facioscapulohumeral Muscular Dystrophy (FSHD)

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common muscular dystrophies in adult and so far there is no curative or preventive treatment. FSHD is characterized by a loss of repressive epigenetic marks within the D4Z4 array located on the subtelomeric part of chromosome 4 , leading to chromatin relaxation and, when associated with a permissive chromosome 4, to the expression of the normally silenced DUX4 protein, whose open reading frame is present in each D4Z4 repeat. DUX4 is a transcription factor resulting in a poison protein through induction of downstream genes. Several pathways have been described as modulated by DUX4, ultimately leading to myofiber death. Here, we designed an AAV vector carrying a shRNA directed against DUX4 (AAV-shDUX4) to knock down DUX4 expression both in FSHD muscle cells and in the cre-inducible DUX4 bi-transgenic mouse model (ACTA1-MCM/FLExDUX4) that expresses high levels of DUX4 after tamoxifen injection. FSHD primary cells were transduced with a lentiviral vector containing the shDUX4. A downregulation of DUX4 mRNA was observed (up to 80%), showing the efficacy of the shRNA.

Next, ACTA1-MCM/FLExDUX4 mice were injected with either an AAV-shScrambled or an AAV-shDUX4 n the presence of tamoxifen. In the shDUX4-treated animals, a reduced activation of the genes downstream DUX4 (95% inhibition for Wfdc3, 88% for Ilvbl, 66% for Sord1), a reduced expression of the genes related to fibrosis (42% inhibition for Pdgfra, 66% for Col3a) and increased levels of myostatin (+80%) were observed at mRNA levels. The pathological signs of the disease were also decreased with a reduced number of re-generating fibres, less fibres with centrally located nuclei and a reduced inflammation. These results support an AAV-shDUX4 approach as a treatment for FSHD.

670 Systemic AAV9-*IGHMBP2* Gene Therapy Administration Ameliorates Disease Phenotype in Symptomatic Spinal Muscular Atrophy with Respiratory Distress Type 1 (SMARD1) Mice

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Spinal Muscular Atrophy with Respiratory Distress type 1 (SMARD1) is a rare autosomal recessive motoneuron disease with infantile onset, with an estimated incidence of 1:100'000. It is caused by mutations in the *immunoglobulin mu-binding protein 2* (*IGHMBP2*) gene, which lead to a deficient amount of the encoded protein. The main clinical symptoms are distal muscular atrophy, diaphragmatic palsy and cardiac hypertrophy. Currently no therapies are approved. Recently, gene therapy emerged as a promising tool for monogenic disease as SMARD1. In our lab we already confirmed the efficacy of AAV9 vector therapy carrying the wild type copy of the *IGHMBP2* gene in SMARD1 mice model when administered pre-symptomatically at postnatal day 1 with both local or systemic injection. Our results participated to pave the way for the first Phase I/IIa gene therapy-based clinical trial for IGHMBP2-related disease started in December 2021 at Nationwide Children's Hospital, Columbus, Ohio. An important question in the field relates to the temporal requirements for this treatment. To examine the therapeutic window, in our work we tested the efficacy of a CAG-AAV9-*IGHMBP2* vector administered in already symptomatic SMARD1 mice. Expression analysis on treated mice demonstrated the ability of the construct in increase the IGHMBP2 protein expression level resulting in an extended survival time, and a partial improvement in phenotype and motor behaviours. No amelioration were observed in typical neuropathological hallmarks of the disease as motor neuron degeneration and muscular innervation even if an unexpected significative rescue of spinal cord astrocytosis and microglia overactivation were detected. In addition we demonstrated also a recovery of muscles fibers' diameter in gastrocnemius and a significative reduction of fibrosis in the heart. A parallel analysis of IGF1 axis involved in cardiac growth and contractility, already demonstrated altered in SMARD1, confirmed that our treatment was able to rescue this dysregulation. Overall, our results suggested that the delayed treatment was efficacious in partially rescue the pathological phenotype of symptomatic SMARD1 mice, leading us to speculate that the effects were more peripheral than acting at the central nervous system level, providing major benefits in the muscular compartment and in spinal cord neuroinflammatory microenvironment. These results support the possibility of a clinical translation of the treatment also during the symptomatic phase of the disease, and suggest the relevance of peripheral impairment in the progression of the pathology, opening future perspectives to the potentiality of a combined therapy targeting multi-organ defects.

671 A Novel Humanized Knock-In Mouse Modeling a Deep Intronic Mutation in Collagen VI-Related Dystrophy Has Weaker Grip Strength and More Centrally Nucleated Myofibers

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Collagen VI-related dystrophies (COL6-RDs) are a group of congenital and early-onset muscular dystrophies characterized by progressive muscle weakness and joint contractures leading to loss of ambulation. COL6-RDs are caused by deleterious variants in any of the genes (*COL6A1*, *COL6A2*, and *COL6A3*) that encode the 3 major α chains of collagen VI, a critical component of the myomatrix. A common recurrent dominant-negative deep intronic C>T variant in the *COL6A1* gene inserts a 72-nucleotide-long pseudoexon between exons 11 and 12 in 25% of the total *COL6A1* mRNAs in heterozygote cells. This variant causes the severe Ullrich congenital muscular dystrophy form of COL6-RDs. To study the pathomechanisms of this variant and to test splice-correction therapies *in vivo*, we have generated a humanized knock-in mouse model carrying the human *COL6A1* wild type (HumC) and/or mutant (HumT) alleles using CRISPR/Cas9. 25% of the *Col6a1* mRNAs transcribed by the *Col6a1*$^{HumT/HumT}$ mice contain the pseudoexon. Here, we characterized the functional and histological phenotypes of this new model and identified outcome measures that will enable assessing the efficacy of therapies in rescuing phenotype. Grip strengths and weights of male and female *Col6a1*$^{+/+}$, *Col6a1*$^{+/HumC}$, *Col6a1*$^{HumC/HumC}$, *Col6a1*$^{+/HumT}$, *Col6a1*$^{HumC/HumT}$, and *Col6a1*$^{HumT/HumT}$ mice were measured monthly from postnatal day (PND) 28 to PND 140 ($n \geq 4$). Additionally, the inverted screen test hanging times of *Col6a1*$^{HumC/HumC}$, *Col6a1*$^{HumC/HumT}$, and *Col6a1*$^{HumT/HumT}$ mice were measured at PND 56, 84, and 112. Muscles from 11-month-old *Col6a1*$^{+/+}$, *Col6a1*$^{+/HumC}$, and *Col6a1*$^{+/HumT}$ mice were stained for histological assessment. The grip strengths of *Col6a1*$^{HumC/HumT}$ and *Col6a1*$^{HumT/HumT}$ males were significantly weaker than *Col6a1*$^{+/+}$ and other HumC males at 1, 3, and 4-month-old ($p<0.05$). 3-5-month-old *Col6a1*$^{HumC/HumT}$ females were significantly weaker than *Col6a1*$^{+/+}$ and other HumC groups. *Col6a1*$^{HumT/HumT}$ females were significantly weaker than all non-HumT females at all tested ages. The weights of *Col6a1*$^{HumC/HumT}$ males were significantly lower than *Col6a1*$^{HumC/HumC}$ at 2 months and lower than *Col6a1*$^{+/HumC}$ males at 2 and 3 months. There was no significant difference between the weights of all other genotypes. The hanging times of *Col6a1*$^{HumC/HumT}$ males were significantly

shorter than those of *Col6a1*$^{HumC/HumC}$ at 2 and 3 months of age. The hanging times of 3 and 4-month-old *Col6a1*$^{HumC/HumT}$ females were significantly shorter than *Col6a1*$^{HumC/HumC}$ females. The differences between the hanging times of all other groups were not statistically significant. Our preliminary histological data indicate that the quadriceps of *Col6a1*$^{+/HumT}$ mice had more internalized nuclei than the other genotypes' quadriceps. Furthermore, the tibialis anterior and quadriceps muscles of *Col6a1*$^{+/HumT}$ mice had more internalized nuclei than the extensor digitorum longus, gastrocnemius, soleus, and triceps muscles. Our findings show that central nuclei quantification and grip strength measurement of this mouse model could be appropriate phenotypic outcome measures to assess splice-correction therapies. The generation of humanized mouse models is an effective tool for the preclinical study of splice-modulating therapeutics directed at human disease-causing variants.

672 Developing Cas13-ADAR-Mediated *DUX4* mRNA Editing as a Prospective Therapy for FSHD

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Facioscapulohumeral Muscular Dystrophy (FSHD) is among the most prevalent muscular dystrophies, affecting over 830,000 people worldwide. FSHD is an autosomal dominant or digenic disorder caused by de-repression of the toxic *DUX4* gene in skeletal muscle. DUX4 encodes a transcription factor that is normally active during embryogenesis but otherwise off in skeletal muscle. Currently no treatment exists that alters the course of FSHD, and therapy development remains an unmet need in the field. We propose that the most direct route to FSHD therapy will involve *DUX4* inhibition. Numerous labs in the FSHD field, including ours, are developing various strategies to inhibit *DUX4* using different mechanisms. Here we describe an approach to silence *DUX4* mRNA using new CRISPR/Cas13-based RNA editing strategy. Cas13 was originally developed as a RNA-guided CRISPR enzyme that cleaves RNA and not DNA. In addition, modified Cas13 can also be employed to precisely edit single bases on mRNAs. To do this, Cas13 is fused to a modified ADAR2 sequence (Adenosine Deaminase Acting on RNA) to produce a fusion protein that can direct cytidine-to-uridine editing on target mRNAs (C-to-U). Using this approach, glutamine and arginine codons can be edited to stop codons, thereby producing truncated, potentially non-toxic *DUX4* open reading frames. This precision editing requires the use of guide RNAs. Because this technology is new, guide RNA design still requires optimization. Here we developed a high-throughput dual reporter system to test Cas13/ADAR-mediated RNA editing of *DUX4* mRNAs. Our strategy involved developing complex stable cell lines expressing doxycycline-inducible Cas13/ADAR; a transcriptionally active but non-toxic *DUX4* ORF; and NanoLuciferase reporter gene driven by a DUX4-induced promoter. Thus, NanoLuc is expressed in the presence of DUX4 and reduced or absent with DUX4 knockdown treatments. We designed 117 different guide RNAs targeting 34 *DUX4* glutamine and 5 arginine codons, with the goal of editing premature STOP codons. To date, we have identified several DUX4-modifying gRNAs that caused significantly reduced NanoLuc expression, suggesting successful editing of *DUX4* mRNA. Our results may provide another treatment option for FSHD, and has implications for using this system to edit other transcripts. We will discuss prospects for uncovering better "rules" for designing effective RNA-editing gRNAs.

673 Antibody-Mediated Delivery of AAV9 Enhances Skeletal Muscle Targeting and Therapeutic Efficacy in Mouse Models of Genetic Muscle Disease

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The use of adeno-associated virus (AAV) for the delivery of gene therapies holds great promise for the treatment of many muscle diseases, with recent clinical trials demonstrating improved muscle function following systemic AAV treatment. However, the extremely high systemic doses needed to deliver AAV to all skeletal muscles throughout the body often results in off-target tissue transduction (e.g., liver) and toxicity, with only low amounts of AAV effectively transducing muscle. To overcome this challenge, we have taken a rational engineering approach to design AAVs that are targeted to skeletal muscle via the conjugation of muscle-specific antibodies (Abs). We generated and screened Abs against the skeletal muscle-specific protein, CACNG1, and conjugated these Abs to AAV9 capsids with mutated galactose binding domains. We then packaged therapeutic transgene constructs into these muscle-retargeted capsids, and tested their efficacy in mouse models of disease, including: Duchenne muscular dystrophy (D2-mdx), X-linked myotubular myopathy (MTM1 KO) and limb-girdle muscular dystrophy 2I/R9 (Fkrp^{P448L}). Overall, systemic treatment of diseased mice with our CACNG1 Ab-retargeted AAV9 substantially enhanced therapeutic transgene expression within multiple skeletal muscles while also reducing transduction of the liver when compared to WT AAV9. In D2-mdx mice, higher expression of microdystrophin in the muscle of CACNG1 Ab-retargeted AAV9 treated mice lead to greater reductions in muscle damage (assessed by serum creatine kinase) and improved grip strength compared to WT AAV9 treated mice. In MTM1 KO mice, higher expression of MTM1 in the muscle of CACNG1 Ab-retargeted AAV9 treated mice led to improved muscle histopathology and enhanced survival compared to AAV9 treated mice. Finally, in Fkrp^{P448L} mice, higher expression of FKRP in the muscle of CACNG1 Ab-retargeted AAV9 treated mice led to enhanced glycosylation of α-dystroglycan and improved exercise capacity. Importantly, the functional improvements observed in these studies were achieved using relatively low systemic doses of CACNG1 Ab-retargeted AAV9 (ranging from ~2E+12vg/kg to ~6E+13vg/kg), and compared to WT AAV9, there was a ~95% reduction in transgene expression in the

livers of CACNG1 Ab-retargeted AAV9 treated mice. Additionally, while our CACNG1 Ab-retargeted AAV9 platform was developed to enhance skeletal muscle transduction, we found that by modulating the galactose binding mutations in our capsid, we can skew tropism towards both skeletal muscle and heart, which is beneficial for certain diseases such as Duchenne muscular dystrophy. In summary, these studies have demonstrated that CACNG1 Ab-mediated delivery of AAV9 may be used to enhance the therapeutic efficacy and reduce off-target toxicity of gene therapies in multiple muscle diseases.

674 Sustained Efficacy of CRISPR-Cas13b Gene Therapy for FSHD is Challenged by Immune Response to AAV.Cas13b Vectors

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Facioscapulohumeral muscular dystrophy (FSHD) is potentially devastating muscle disease caused by de-repression of the toxic *DUX4* gene in skeletal muscle. FSHD patients may benefit from *DUX4* inhibition therapies, and although several experimental strategies to reduce *DUX4* levels in skeletal muscle are being developed, no approved disease-modifying therapies currently exist. We developed a CRISPR-Cas13b system that cleaves and eliminates *DUX4* mRNA and protein, protects cells from DUX4-mediated death, and reduces FSHD-associated biomarkers *in vitro*. We found little to no evidence of collateral transcript cleavage or significant off-target effects in human myoblasts *in vitro*. *In vivo* delivery of the CRISPR-Cas13b system with adeno-associated viral vectors (AAV6) reduced acute damage caused by high *DUX4* levels in a mouse model of severe FSHD. However, protection was not sustained over time, with reductions in Cas13b and guide RNA levels between 8 weeks and 6 months after injection. In addition, wild-type mice injected with AAV.Cas13b showed muscle inflammation with infiltrates containing AAV.Cas13b-responsive CD8+ CD4+ cytotoxic T cells. Our findings suggest successful *in vivo* implementation of CRISPR/Cas13-based gene therapies may require strategies to mitigate immune responses.

675 iTenocytes on Microgrooved Scaffolds Promotes Achilles Tendon Regeneration

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50% of musculoskeletal injuries involved tendons and ligaments, which have a poor innate healing capacity. Full structure and function restoration after injury remains a major unmet clinical need. Developing a tissue engineered tendon could dramatically alter patient outcomes, yet current methods fall short in terms of biomechanical performance and organization. Previously, we have shown the tenogenic potential of iPSC-derived mesenchymal stromal cells (iMSCs), stimulated by scleraxis (SCX) overexpression (iMSC^{SCX+}). We have developed a 3D-printed polycaprolactone (PCL) scaffold (Fig. 1a) with uniaxial microgrooves that induces end-to-end cellular alignment, mimicking cues found in developing tendons. The goal of this study was to explore the potential of iMSC^{SCX+} seeded onto 3D printed microgrooved PCL scaffolds to improve tendon regeneration in a rat model of Achilles tendon defect repair. Non-patterned scaffolds (NPS) and microgrooved scaffolds (MS) were 3D printed through microgrooved and non-patterned nozzles. Scaffolds were imaged using SEM and tested for printing consistency and biomechanical properties. Human iPSCs were differentiated to MSC lineage using our published protocol (iMSCs) and lentivirally transduced with SCX-GFP. NPS and MS were seeded with iMSC^{SCX+} and cultured to assess cell alignment and gene expression. iMSC^{SCX+} seeded MS were tested in a rat model of Achilles tendon defect with the following groups: 1) Suture only, 2) MS only, and 3) MS+iMSC^{SCX+}. An Achilles defect was surgically induced and either sutured or repaired with MS only or with iMSC^{SCX+} seeded MS (Fig. 2a). For follow-up, gait analysis was performed. At 6 weeks post-surgery, the tendons were harvested for biomechanical testing and histological and immunofluorescent (IF) staining. SEM image analysis (Fig. 1e) showed consistency in scaffold filament size from 3D printing (Fig. 1b). Biomechanical testing of the scaffolds showed that MS had biomechanical properties comparable to native tendon (Fig. 1c). Gene expression analysis of iMSC^{SCX+} seeded on MS or NPS *in vitro* revealed a significant upregulation of tendon marker expression in the iMSC^{SCX+} grown on MS compared to 2D culture or NPS (Fig. 1d). These results were further validated by IF with cells appearing more linearly organized on the MS compared to the NPS (Fig. 1e and 1f). *In vivo,* gait analysis found that the MS+iMSC^{SCX+} group had significant improvements in functional gait recovery compared to the control groups (Fig. 2a). Biomechanical testing of the tendons showed a significant improvement in biomechanical properties in the MS+iMSC^{SCX+} group compared to that of the Suture and MS only groups (Fig. 2b). Regular tissue formation in MS+iMSC^{SCX+} group seemed to be observed in histology (Fig. 2b) and IF stainings (Fig. 2c). This study shows the potential of iMSC^{SCX+}-seeded microgrooved scaffolds as a viable alternative for current tendon defect treatments.

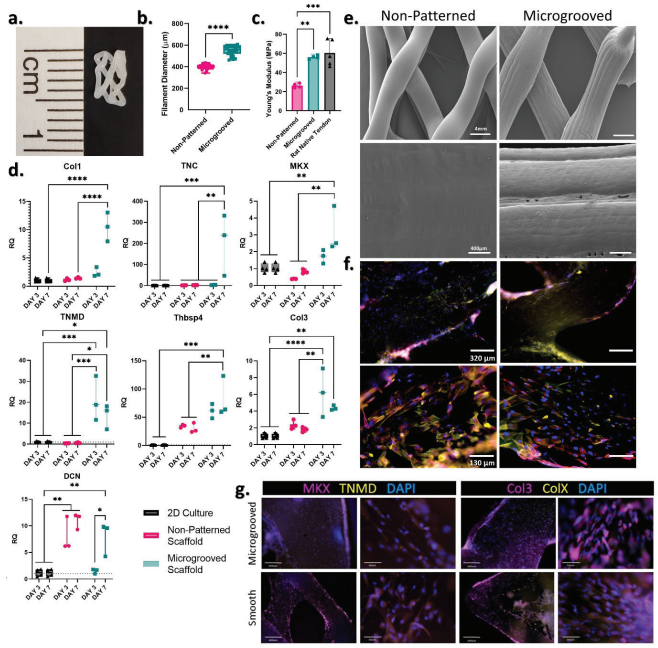

Fig 1: Microgrooved 3D printed scaffolds has biomechanical properties comparable to native tendon and increases tenogenic expression in iMSC^{SCX+}. (a) Scaffold macro-topography. (b) Filament diameter. (c) Scaffold vs native tendon elasticity. (d) Scaffold gene expression. (e) SEM images of non-patterned and microgrooved scaffold. (f) Cellular alignment imaging. (g) ICC staining of scaffold at 4x and 20x.

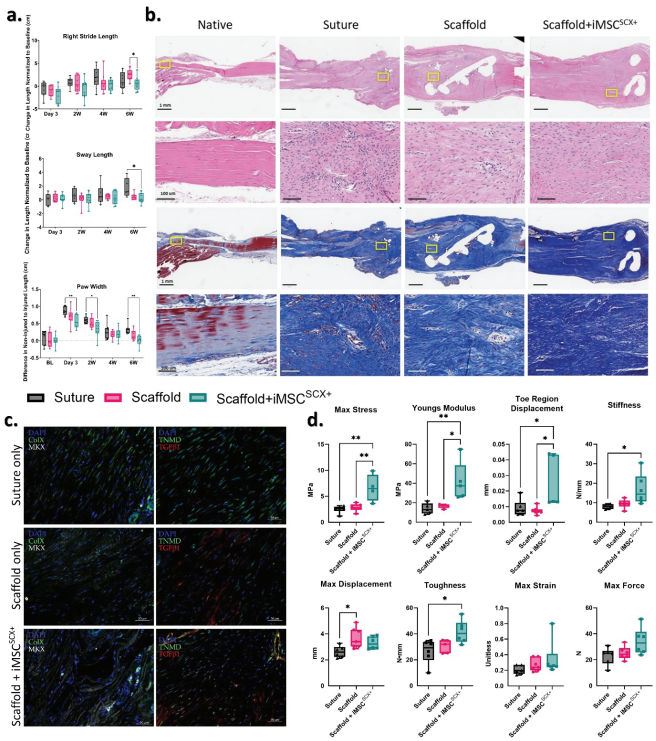

***Fig 2*: iTenocytes-seeded microgrooved scaffolds to regenerate Achilles tendon defect in rats.** *(a) Gait analysis. (b) H&E (top) and Massons Trichome staining (bottom) of Achilles tendon. (c) IF staining of Achilles tendon. (d) Biomechanical testing results.*

676 Investigation of Mortalin's Role in Aberrant Scar Formation Using Human Dermal Fibroblasts and Rat Incisional Scar Model

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Wound healing is a complex multistep process, and dysregulation of this process causes formation of pathologic scars. In present report, we investigated the role of chaperone protein, mortalin, in scar formation and its interaction with interleukin-1α receptor using in vitro and in vivo scar formation models. A recombinant adenovirus expressing short hairpin RNA against mortalin (dE1-RGD/GFP/shMot) was generated and its effect on dermal fibroblasts were evaluated by assessment of cell viability, qRT-PCR, western blotting, immunofluorescence, and immunoprecipitation studies. An incisional wound model using rats were utilized to evaluate the effect of dE1-RGD/GFP/shMot on scar formation. *In vitro*, the mortalin-treated human dermal fibroblast displayed a significant increase in proliferation of type I collagen, α-smooth muscle actin, transforming growth factor-β, phospho-Smad2/3-complex, and NF-κB expression levels. Further, immunofluorescence staining revealed markedly increased mortalin and interleukin-1α receptor protein in keloid tissues compared to those of nonpathological normal tissues, demonstrating that mortalin and interleukin-1α receptor were responsible for the aberrant fibrosis of keloid tissues. *In vivo*, dE1-RGD/GFP/shMot treatment significantly decreased the scar size and type-I-collagen, α-SMA, and phospho-Smad2/3-complex expression levels in rat incisional scar tissues. Together, our findings demonstrate that mortalin and interleukin-1α contribute to aberrant fibrosis during wound healing process, and suppression of mortalin expression level by dE1-RGD/GEP/shMot can effectively inhibit the TGF-β/α-SMA axis and NF-κB signal pathways to prevent aberrant fibrosis during scar formation.

677 Use of Circular RNA Guides to Correct a Hotspot of Dominant-Negative Glycine Substitutions Causing Collagen VI-Related Dystrophies

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Collagen VI-related dystrophies (COL6-RD) are a group of congenital neuromuscular disorders for which there is no effective treatment. Dominant-negatively acting pathogenic variants, in particular glycine substitutions of the conserved collagenous Gly-X-Y motives, are common in the triple helical domains of the *COL6A1*, *COL6A2* and *COL6A3* genes. By virtue of their specific position in the triple helical domain they are capable of incorporating into the hierarchical assembly of the collagen α1, α2 and α3 (VI) chains and as a consequence, produce a dysfunctional collagen VI extracellular matrix. Two of the most common Gly substitutions, p.G284R and p.G293R are located in the alpha1(VI) chain, in a region that constitutes a hotspot for dominant-negative mutations. Most of glycine missense pathogenic variants are caused by a single guanosine (G) to adenosine (A) change, making them suitable for the use of an RNA editing approach using endogenous adenosine deaminases (ADARs). ADARs use double-stranded RNAs as a substrate to catalyze adenosine (A)-to-inosine (I), which mimics

G during translation. Here, we investigate the use of circular ADAR-recruiting RNAs to recruit endogenous ADAR enzymes to change a specific A to I, as a therapeutic approach for collagen VI-related dystrophies caused by G>A changes. RNA circularization improves RNA stability and increases resistance to cellular exonucleases, improving the efficiency and durability of programmable RNA editing. Strikingly, preliminary results in HEK293T cells with fluorescence reporters for both the p.G284R and p.G293R alleles showed percentages of RNA editing up to 22% and 25%, respectively, using a single circular ADAR-recruiting RNA to address both variants. This approach will be tested *in-vitro* in patient-derived fibroblasts and *in-vivo* in humanized mouse models we are generating.

678 Nuclease-Dead Cas9-Mediated Downregulation of alpha-Synuclein as a Disease-Modulatory Therapeutic Strategy for Parkinson's Disease

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Parkinson's disease and related alpha-synucleinopathies show growing numbers due to an aging society. However, no approved disease modulatory therapies exist that halt disease progression. Alpha-synuclein is a critical therapeutic target that is found as an aggregated form in Lewy bodies, the diagnostic hallmark of Parkinson's disease, and mere overexpression of alpha-synuclein in hereditary conditions leads to neurodegeneration. Here, we describe the development of a nuclease-dead S. aureus Cas9 (sadCas9) CRISPR interference (CRISPRi) system to downregulate alpha-synuclein in 1) a human induced pluripotent stem cell (iPSC)-derived neuronal model and 2) a transgenic humanized alpha-synuclein mouse model. The mechanism of action is based on the principle that a complementary single guide RNA (sgRNA) recruits sadCas9 mutant protein to the promoter region of alpha-synuclein and inhibits transcription resulting in reduced target gene expression. We want to test whether this approach has the potential to become a therapeutic strategy for disease modification in Parkinson's disease. We designed and screened 32 sgRNAs across the human *SNCA* promoter and identified several sgRNAs that mediate the downregulation of alpha-synuclein at varying levels in HEK cells and human iPSC models. CRISPRi downregulation of alpha-synuclein in patient iPSC-derived neuronal cultures showed reduced oxidative stress levels and mitochondrial DNA damage. Next, we tested a lead sgRNA in a humanized alpha-synuclein mouse using an all-in-one AAV9 construct from which we expressed sadCas9 and the lead sgRNA. Four months old mice were stereotactically injected with the AAV9 lead or control sgRNA and euthanized 1 or 6 months after surgery. We show robust expression of sadCas9 and reduction of alpha-synuclein (mRNA and protein) after unilateral stereotactic injection into the substantia nigra of adult mice. Initial microglia activation after one month subsided, and microglia marker Iba1 and CD16/32 did not significantly differ at six months between lead sgRNA and control conditions. We conclude that Cas9 interference technology facilitates alpha-synuclein mRNA and protein reduction in *vitro* and *in vivo*. This pre-clinical work shows promise in advancing to a regenerative medicine-based therapeutic genetic therapy for Parkinson's disease or related alpha-synucleinopathies.

679 Gene Therapy Using a CNS Dominant Human FMRP Isoform Rescues Seizures, Fear Conditioning, and Sleep Abnormalities in Fmr1 KO Mice

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Fragile X Syndrome (FXS) is a monogenic neurodevelopmental disorder caused by the loss of expression of the fragile X mental retardation protein (FMRP), a key mRNA binding protein required for brain function. FXS is the most prevalent single gene cause of autism and intellectual disability in males, affecting approximately 1:5000 men and 1:8000 women. Viral vector-mediated gene replacement therapy has been studied in the past as a potential viable treatment for the disorder. Although partial rescue of abnormal behaviors has been reported in Fmr1 KO mice and rats, complete rescue of the abnormal phenotypes has not been achieved, including certain salient features such as audiogenic seizures in Fmr1 KO mice. Here, the distribution pattern, therapeutic effects, and safety profile of a novel self-complementary adeno-associated viral (AAV) vector containing a major human brain isoform of FMRP were studied following intrathecal injection into neonatal wild-type and Fragile X KO mice at a clinically relevant dose. Widespread FMRP expression was observed throughout the brain at 1, 3 and 6 months after injection in AAV-treated KO mice. Expression was primarily in neurons with sparse expression in glia, similar to the endogenous expression profile in uninjected WT mice. In behavioral tests, AAV-treated KO mice showed robust suppression of audiogenic seizures and normalization of fear conditioning behavior. Multi-day monitoring of circadian locomotor activity revealed normalization of hyperactivity and sleep, while electroencephalographic recordings showed reversal of slow wave deficits. Further assessment of individual transgene expression and behavioral responses demonstrated correlations between the level of FMRP expression and drug efficacy. No significant adverse effects were observed in behavioral, serological or pathohistological markers up to 12 months after intrathecal injection into WT adult mice. These preclinical findings represent our most comprehensive assessment of AAV-mediated gene therapy in Fmr1 KO mice to date and further demonstrate the validity of its use for treating the most common genetic cause of cognitive impairment and autism in children.

680 AAVrh10 Mediated Gene Replacement Therapy Targeted to Schwann Cells to Treat CMT1X Demyelinating Neuropathy

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Introduction In previous work we demonstrated the efficacy of two different AAV serotypes, AAV9 and AAVrh10, to target the peripheral nervous system (PNS) and to transduce myelinating Schwann cells for the treatment of demyelinating neuropathies. X-linked Charcot Marie-Tooth disease type 1 (CMT1X) is the second most common form of CMT neuropathies resulting from loss of function mutations in the *GJB1* gene, which encodes the gap junction protein connexin 32 (Cx32). Cx32 is highly expressed in myelinating Schwann cells throughout the PNS forming gap junctions in non-compact myelin areas. To develop a gene replacement therapy for CMT1X, we performed a dose-response treatment trial using the AAVrh10 serotype to deliver the human *GJB1* gene into the *Gjb1*-null mouse model of CMT1X, under the control of the Schwann cell specific human myelin protein zero (MPZ) promoter. **Methods** AAVrh10-*MPZ.Egfp* (mock) and AAVrh10-*MPZ.GJB1* (therapeutic) vectors were delivered by lumbar intrathecal injection in wild type or *Gjb1*-null mice, respectively. The mock vector was tested at the dose of $2x10^{11}$ vg for biodistribution and EGFP expression, while the therapeutic vector was tested in three different doses ($1x10^{11}$, $2x10^{11}$, and $1x10^{12}$ vg) in order to evaluate biodistribution and Cx32 expression in PNS tissues. We then proceeded with a dose-escalation treatment trial in 2-month-old *Gjb1*-null mice and evaluated functional and morphological outcomes compared to mock-vector treated littermates. Functional analysis included behavioral testing at 2- and 4-months post-injection while electrophysiological and morphological analysis was conducted 4 months post-injection. **Results** Biodistribution and expression analysis of the mock vector showed widespread targeting of the PNS with vector genome copy numbers (VGCNs) reaching 3.3±0.79 in lumbar roots, 2.7±1.38 in proximal sciatic nerves and 0.1±0.02 in the distal parts of the sciatic nerve. EGFP expression rates reached 24.0±1.64 % in lumbar roots and 49.5±4.54 % in sciatic nerves. Biodistribution and expression analysis of the therapeutic vector showed a dose-dependent Schwann cell transduction with most efficient targeting of peripheral nerves in mid- and high-dose treated *Gjb1*-null mice. Cx32 expression rates in PNS Schwann cells of low-dose treated mice ranged between 23.5-30.7%, whereas they were significantly higher in PNS tissues of mid- and high dose treated animals, ranging between 72.1 and 82.6%. Functional outcomes showed improved grip strength and sciatic nerve motor conduction velocity in animals injected with the standard and high vector dose, but not with the low dose. Furthermore, in both anterior lumbar roots and peripheral nerves, we observed a statistically significant reduction of around 50% in morphological parameters evaluated including the ratio of abnormally myelinated fibers and the number foamy macrophages, in all three treatment groups (low, standard and high-dose AAVrh10-*hMpz-GJB1* groups) compared to the mock-treated controls. **Conclusion** This study demonstrates that a single lumbar intrathecal injection of clinically relevant doses of AAVrh10-*MPZ.GJB1* vector leads to adequate biodistribution to the PNS and high rates of Schwann cell-specific therapeutic gene expression, as well as significant therapeutic benefit evaluated by functional and morphological outcomes in the model of CMT1X demyelinating neuropathy.

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681 Identifying Potential Clinical Outcomes for Future Clinical Trials for SLC13A5 Deficiency

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Cellular metabolism is a complex process required for organisms to maintain life. Disruptions to citrate transport caused by biallelic loss of function variants in the *SLC13A5* gene leads to a rare genetic neurodevelopmental disorder called SLC13A5 deficiency (i.e. SLC13A5 Epilepsy, Citrate Transporter Disorder, Developmental Epileptic Encephalopathy-25). Gene therapy presents an opportunity to target the root cause of monogenic disorders such as SLC13A5 deficiency, and there is an AAV9-based gene therapy for this in preclinical development. Patients present with infantile epilepsy, severe motor impairments, limited language abilities, disruptive sleep problems, and teeth hypoplasia. Individuals typically present with seizures within the first few days of life, often have recurrent episodes of status epilepticus, and have severe developmental disability requiring lifelong dependence on caregivers. Treatment is currently limited to supportive care to manage seizures and other disease manifestations, and there is an unmet need to address the developmental aspects of the disorder. It is important to establish a disease concept model that characterizes the disease burden and most significant challenges. Caregiver perspective contributes to improved disease understanding and the identification of clinical outcomes that are meaningful for the affected individual and their caregivers. We conducted a cross-sectional survey of SLC13A5 deficiency caregivers with both Likert scale and free responses. Surveys were distributed to English-speaking families through the TESS Research Foundation caregiver network. Since this is an inherited disorder that was discovered in 2014, several caregivers have 2 or 3 affected children. Twenty-five caregivers submitted a total of 27 responses that each represent a unique affected child. The objectives of this qualitative and quantitative survey were to define the goals and concerns of the caregivers, and to describe the experience of caring for an individual with SLC13A5 deficiency. The symptoms identified as severely impactful by the caregivers were speech impairments (70.4%), motor disability (63.0%), cognitive impairments (48.1%), and seizures (48.1%). Differences in how SLC13A5 deficiency affects patients and caregivers were apparent between patient age groups. Caregivers of individuals younger than 7 years reported seizures and motor disability as major concerns, while those with older dependents focused more on developmental disability that limited their independence. Meaningful clinical outcomes identified include the affected individual gaining the ability to walk, dress, toilet, eat, speak, or read, as well as reductions in seizure frequency and severity for those with uncontrolled seizures. SLC13A5 deficiency is a primary epilepsy, yet there are other aspects of this disease that are important to patients and their families. In addition to seizure control, success for a gene therapy for SLC13A5 deficiency would demonstrate improvements in mobility, cognition,

and communication. Future clinical trials for this disorder should consider outcome measures that capture these symptoms. This study demonstrated that families of people affected by SLC13A5 deficiency value a broad spectrum of clinical outcomes, demonstrating the importance of including their perspective in therapeutic development.

682 Engineered Zinc Finger Transcriptional Regulators Enable Robust and Reliable Epigenetic Regulation in the Mouse Brain

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Genomic medicines have the potential to transform the treatment of neurodegenerative and neurodevelopmental diseases. Towards that end, we are developing zinc finger transcriptional regulators (ZF-TRs) to therapeutically modulate genes underlying neurological disorders. ZF-TRs are derived from naturally occurring human proteins and can be engineered to recognize specific genomic DNA sequences. Uncovering novel regulatory sites for highly specific epigenetic regulation (repression or activation) can require extensive empirical testing. Using our proprietary design algorithm, we generated hundreds of candidate ZF-TRs against multiple neurological disease targets to efficiently identify regulatory hot spots in both mouse and human genomes. We then developed an iterative and automated *in vitro* screening platform to test their on- and off- target activity in cell lines and cultured neurons before assessing their performance in animal models. In the first step of this workflow, we transiently transfected mRNA for hundreds of candidate ZF-TRs per target per species at multiple doses into human (SK-N-MC) or mouse (Neuro-2a) neuroblastoma cell lines. After 24 hours, we performed automated RT-qPCR analysis to assess changes in target gene expression. For each target, we identified unique regulatory sites permissive to regulation by a collection of active ZF-TRs. From these candidates, we selected dozens per target per species and manufactured AAV-ZF-TR vectors to examine their activity in primary (mouse) or iPSC-derived (human) neurons. We observed that ZF-TR performance in neurons was highly correlated with that in neuroblastoma lines. Notably, most of the ZF-TRs screened in neurons demonstrated potent and dose-dependent target gene regulation. Next, we evaluated AAV-ZF-TR specificity using high-throughput Affymetrix microarray analysis to examine the number of differentially expressed genes between ZF-TR and control-treated neurons. Using this approach, we identified several active ZF-TRs per target with few to no detectable off-targets. To confirm their activity *in vivo*, we delivered a subset of mouse-targeted AAV-ZF-TRs to wildtype adult mice and measured their impact on target gene expression in multiple brain regions by RT-qPCR analysis. All ZF-TRs tested showed robust target reduction in each of the brain regions examined. Our ability to engineer potent and selective ZF-TRs targeting novel regulatory sites for multiple targets and species highlights the potential of this epigenetic regulation platform to create genomic medicines for an array of nervous system diseases.

683 Too Much or Too Little: AAV-Based Gene Replacement Therapy for *SURF1*-Related Leigh Syndrome

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SURF1(surfeit locus protein 1)-related Leigh syndrome (LS) is an early onset neurodegenerative disorder characterized by reduction in the assembly factor of complex IV, resulting in disrupted mitochondrial function. Patients with this disease show degenerations in multiple brain regions, including basal ganglia, diencephalon, brain stem and spinal cord. Here, we hypothesized that a functional gene replacement strategy could restore mitochondrial functions in LS caused by *SURF1* loss-of-function mutations. We have previously reported a codon-optimized version of the human *SURF1* (h*SURF1*) packaged within self-complementary adeno-associated virus serotype 9 (AAV9) viral vectors (AAV9/h*SURF1*) to drive strong and ubiquitous expression of *SURF1* in all organs (Vector-A), including the central nervous system (CNS). Our study suggested that Vector-A was effective in restoring abnormalities in the *SURF1* KO mice, and was safe in the WT mice for at least a year. However, the same vector led to inflammation and degeneration in multiple tissues of WT adult rats, including heart, liver, and dorsal root ganglion, most notably at 30 days post-dosing. In order to minimize the toxicity while maintaining the efficacy of the gene therapy, we modified the design of Vector-A to reduce the expression of *SURF1*, which led to the design of Vector-B, Vector-C and Vector-D. We compared the effectiveness of all four vectors in restoring complex IV activity in patient fibroblasts, which showed similar degrees of improvement. We further proceeded to test Vector-D (the vector that induces the lowest *SURF1* expression) in the *SURF1* KO mice. Our data suggested that Vector-D was able to improve COX activity in different tissues depending on the treatment age, dose and administration route, and it is safe in the WT rats. Our results further suggest that SURF1 overexpression may induce mitochondrial stress. Our ongoing studies involve optimizing the design of our transgene cassette to achieve ideal restoration of SURF1 in all tissues with a treatment scheme that can be safely and effectively translated to patients.

684 AAV1.NT3 Gene Therapy Ameliorates the Clinical Severity of Experimental Autoimmune Encephalomyelitis (EAE) Mouse through Immunomodulation

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Introduction: Multiple sclerosis (MS) is a common demyelinating disease, affecting more than 2.5 million patients worldwide. MS is an autoimmune disease in which the patients' immune system attacks the myelin of the nerves in the brain and spinal cord, eventually causing demyelination and secondary axonal loss. The main causes of MS remain largely unknown, and there is currently no cure for the disease. Neurotrophin 3 (NT-3) is an important growth factor stimulating glial cell survival and differentiation, and stimulates axon growth and myelination. NT-3 is also known to have immunomodulatory and anti-inflammatory properties as we previously demonstrated in the

spontaneous autoimmune peripheral polyneuropathy (SAPP) mouse model for chronic inflammatory polyradiculopathy (CIDP) in humans. In this study, we used the chronic relapsing experimental autoimmune encephalomyelitis (EAE) mouse model to mimic MS disease and investigated the possibility of using AAV1.tMCK.NT-3 gene therapy, via intramuscular delivery into the EAE model to attenuate the disease process as a treatment approach. **Methods:** C57BL/6 mice at age of 8-12 weeks were immunized with synthetic myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}, MEVGWYRSPFSRVVHLYRNGK) in complete Freund's adjuvant with Mycobacterium tuberculosis H37RA. For each mouse, 200 ug of MOG_{35-55} peptides and 200 ug H37RA were emulsified in CFA and injected subcutaneously into the flanks, followed by intraperitoneal injection of 400 ng pertussis toxin. A second dose of pertussis toxin was given after 48 h. AAV1.tMCK.NT-3 was delivered via intramuscular injection to the right gastrocnemius muscle (1.0xE11 vg in Ringer's lactate) at 21 days post EAE induction. This approach ensures continuous systemic effect of NT-3 through secretion from muscle into circulation. Mice were closely monitored using a clinical scoring system and functional studies and sacrificed at approximately 70 days post EAE induction. **Result:** Our data showed that AAV1. NT-3 gene therapy ameliorated the clinical severity of EAE. The clinical score showed substantial improvement of treated mice compared to untreated mice. The treated cohort performed significantly better in rotarod performance and grip strength tests compared to untreated counterparts. These data correlated with reduced expression of the pro-inflammatory cytokines, interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6) in both brain and spinal cord, in the NT-3 treated cohort. The treatment also increased the percentage of $CD3^+CD4^+CD25^+$and $Foxp3^+$ regulatory T cells (Tregs) in the spleens and lymph nodes of both male and female mice. When challenged by myobacterium, the dendritic cells from bone marrow of NT-3 treated mice also showed lower induction of TNF-α. **Conclusion:** In summary, our study demonstrated that AAV1.NT-3 gene therapy reduced the severity of EAE model of MS, possibly through modulation of the immune system. We show that circulating NT-3, following the transduction of muscle via AAV1.tMCK.NT-3 vector injection triggers tolerogenic immune responses by reducing the inflammation and increasing Treg cells in both spleen and lymph node. This proof-of-principle study demonstrates the potential of clinical translational path for AAV-delivered NT-3 for treatment of chronic progressive MS.

685 AAV9-Mediated Gene Therapy in a Knock-In Mouse Model of Infantile Neuroaxonal Dystrophy

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Introduction: Infantile neuroaxonal dystrophy (INAD) is a rare and lethal paediatric neurodegenerative disease. It is caused by biallelic mutations in the *PLA2G6* gene, which codes for the enzyme calcium-independent phospholipase A2. Patients present with progressive neurological symptoms between six months and three years of age, with mortality typically occurring by 10 years old. No disease modifying treatments are available and there is an urgent need to develop new therapies. Methods: We conducted an in-depth characterization of the *pla2g6-inad* knock-in mouse model. Following characterization, we investigated the therapeutic potential of an AAV9.h*PLA2G6* vector administered intracerebroventricularly to neonatal and juvenile *pla2g6-inad* mice. We investigated survival, behavioural parameters, and histological analysis to assess therapeutic efficacy. Results: The average lifespan of the model is reduced to approximately 14 weeks, with weight loss and behavioural decline from 9 weeks old. Neuropathology studies showed neuronal loss and neuroinflammation in the brain and spinal cord, along with autophagic and lysosomal accumulation. A long term-study over 30 weeks demonstrated that neonatally administered AAV9.h*PLA2G6* gene therapy resulted in a significant improvement in all parameters measured including survival, weight, locomotor function, and neuronal counts in both the brain and spinal cord. Furthermore, the autophagic function was restored and lysosomal accumulation was significantly reduced. Adult administrations to symptomatic mice have thus far shown increased survival of 21 weeks on average and improved behavioural function. Conclusion: This study provides novel insights into INAD disease pathology and cellular dysfunction in the CNS and suggests an AAV9-based therapy has potential to enable effective treatment of INAD. Further clinical translation studies are being undertaken with our industrial partner, Bloomsbury Genetic Therapies Ltd.

686 AAV9.CBA.BAG3 Gene Therapy Improves Survival and Function in the SOD1.G93A Mouse Model for ALS

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease caused by the degeneration of upper and lower motor neurons. While majority of ALS cases are sporadic, 10% of the cases are familial. Mutations in superoxide dismutase 1 (SOD1) gene, commonly seen in familial cases, cause production of misfolded proteins which form aggregates in cells, leading to loss of motor neurons. Misfolded proteins can be degraded using ubiquitin-proteasome system or autophagy-lysosome pathway (ALP), depending on several factors, such as state of the misfolding and abundance of aggregates. BCL2-associated athanogene 3 (BAG3) is one the cochaperones in ALP, shown to be upregulated in *in-vitro* and *in-vivo* models with mutant SOD1. In this study, we tested if overexpression of BAG3 mitigates motor neuron death, and consequently increases survival in the SOD1.G93A mouse model for ALS, alone or combined with Neurotrophin-3 (NT-3), a known supporter of neuronal survival. SOD1.G93A mice received AAV9.CBA.BAG3 ($4x10^{12}$ total vg) systemically at 40 (BAG3-40, n=3) and 70 (BAG370, n=4) days of age, prior to prominent intraneuronal aggregate formation (AF), and at day 100 (BAG3-100, n=5) when intraneuronal AF is obvious. The efficacy of circulating NT-3 secreted from muscle was tested with AAV1.tMCK.NT-3 ($1x10^{11}$ total vg) gene therapy via intramuscular injection (IM) at day 40 (NT-3-40, n=3), or as

part of the combinatorial therapy where both vectors were administered at day 40 (Combo-40, n=4) and day 70 (Combo-70, n=9). Ringer's lactate injected cohort (n=7) served as untreated (UT) control group. Mice were tested on rotarod and for grip strength weekly, starting at the time injection, and were perfused at endpoint with formalin. Anterior horn neurons were quantified in cresyl violet-stained cross sections. Survival of SOD1.G93A mice increased significantly in BAG3-100, NT-3-40, and Combo-70 cohorts. The mean age of death (days) was 141.9 ± 2.7 for UT, while it increased to 155.8 ± 2.0 in BAG3-100 (p=0.0073), 160.7 ± 1.3 in NT-3-40 (p=0.0019), and 153.4 ± 2.4 in Combo-70 (p=0.0099) cohorts. BAG3 gene therapy initiated on days 40 and 70, prior to notable AF did not increase the survival compared to BAG3-100 cohort, suggesting that time of BAG3 treatment is critical. BAG3-only cohorts and NT-3-40 performed significantly better in rotarod and grip strength, compared to UT cohort for various time points during the course of the study. Combo-40 surpassed the BAG3-40 and NT-3-40 cohorts, and Combo-70 exceeded the BAG3-70 cohort suggesting that combinatorial treatment approach is more beneficial than BAG3 or NT-3 alone. The quantitative histopathology assessing the number of neurons in the anterior horn area of lumbar segments from each cohort revealed that large neurons (>15 μm) were better preserved in Combo-40, Combo 70, BAG3-100 and NT-3-40 cohorts. We found no further motor neuron loss in these cohorts compared to UT, which died 2 weeks earlier, suggesting that the treatment decreased the natural rate of decline in number of neurons. This study shows that systemic AAV9.CBA.BAG3 gene therapy in the SOD1.G93A mouse model for ALS significantly improved survival and function, when introduced at the time of prominent intraneuronal AF associated with clinical signs of early paralysis. Our combinatorial gene therapy with AAV1.tMCK.NT-3, via IM delivery, further improved the function in this model. We believe this approach might offer therapeutic potential for other protein aggregate-related neurodegenerative disorders.

687 Intracerebroventricular AAV9 Gene Therapy in Neonates Improves Lifespan and Disease Progression over Later Treatment in a Mouse Model of Niemann-Pick Disease, Type C

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Niemann-Pick disease, type C1 (NPC1) is a rare, monogenic neurodegenerative disease affecting approximately 1:100,000 births in the United States causing severe neurological degeneration as well as peripheral symptoms, including hepatosplenomegaly. Though small molecule trials are currently in progress, there are currently no FDA-approved therapies for NPC1. AAV-based gene therapy has been shown to significantly extend lifespans and slow symptom progression in mouse models of NPC1. However, clinical realities may impact the preferred choice of delivery route and age of administration, and the specific relative impacts of treating the central nervous system (CNS) and the periphery has not been clearly shown. To establish the optimal delivery parameters for an efficacious AAV-based gene therapy for NPC1 and determine the relative significance of treating the CNS and peripheral organs, we compared the survival and disease phenotype progression of mice injected with AAV-NPC1 constructs at three different age/delivery route combinations. We found that while all treatments improved outcomes in mice, those treated as neonates via an intracerebroventricular (ICV) injection survived more than 2-fold longer and showed significantly slowed disease progression compared with mice treated at weaning age with either systemic or ICV delivery. We also found that treatment with an AAV8-NPC1 construct that does not reach the CNS had a significantly smaller impact on survival and disease phenotype than all treatments that reached the CNS. However, we observed that splenomegaly, a symptom common in NPC patients but rarely observed in NPC1 mouse models, can in fact appear as well in older neonatal ICV-treated mice. These findings suggest that earlier treatments focused on the CNS are optimal for extending survival and slowing disease progression in NPC1. They also demonstrate that while targeting the CNS is crucial for the most significant measures of disease progression, treatments that reach the periphery may also be important for further improving overall efficacy as well as for addressing liver and spleen pathology, among the most common clinical symptoms of NPC1.

688 Safe and Efficient Intra-CSF Delivery of AAV Vector in Nonhuman Primates

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It has been challenging for adeno-associated virus (AAV) to efficiently transduce central nervous system (CNS) after intravenous (IV) administration, though novel blood-brain barrier (BBB)-crossing serotypes have been actively studied. Therefore, it is still highly needed for clinical applications to identify a safe and effective route of AAV administration to target CNS. To achieve CNS distribution, several ROAs have been applied, such as intraparenchymal, intracerebroventricular, and intrathecal (cisterna, or lumbar). Studies have demonstrated promising results that intrathecal AAV infusion leads to significant gene expression in the CNS, however recent studies conducted in nonhuman primates (NHPs) to compare intra cisterna magna (ICM) and intrathecal lumbar (IT) demonstrated discrepancies. In this study, self-complimentary AAV9-mediated gene expression of green fluorescent protein (GFP) driven by chicken beta actin (CBA) promoter at a dose of $3x10^{13}$ vector genome (vg)/animal was assessed in cynomolgus macaques with necropsy at Day 14 post dosage to compare ICM and IT lumbar administration. We characterized the AAV genome biodistribution by quantitative PCR (qPCR) and GFP protein expression by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) in the CNS and peripheral system following ICM or IT lumbar dosage. AAV distribution and transgene expression (GFP) were high in spinal cord and dorsal root ganglia (DRG) after ICM or IT administration, and there were no significant difference of transgene expression in these tissues or peripheral tissues /nerves between ICM and IT injection. However, the transgene expression was higher in the brain when the AAV was infused in cisterna magna via ICM than lumbar area through IT administration. Intrathecal delivery via lumbar is a convenient dosing route due to the easy access and minimal invasiveness. However, the delivery strategy of ICM administration via suboccipital puncture in NHPs or translation to clinical practice is challenging because of the high risk of severe or

sometimes even fatal complications in animals or patients due to the medullary injury impedes. To overcome this hurdle, we utilized the catheter-mediated cisterna magna delivery strategy by adapting an intravascular microcatheter which can safely navigate in the spinal canal from lumbar section to the cisterna magna under fluoroscopic guidance. In this study, delivery of AAV into cisterna magna area when applied with microcatheter was as safe as IT dosing. No adverse effects were observed during infusion or post-dosage. These results suggest that both ICM and IT lumbar administration of AAV are excellent ROAs when AAV transduction in spinal cord or DRG is needed, whereas if the neurological disease requires brain neuronal transduction, ICM is a better ROA than IT lumbar. Remarkably, with the adaptation of microcatheter, the ICM delivery technique is a safe and minimally invasive alternative to direct infusion into the cisterna magna, achieving the distribution of AAV gene transfer to the brain.

689 Developmental Age Effects for AAV9/SLC6A1 Gene Therapy in a Mouse Model of SLC6A1 Related Disorder

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The human SLC6A1 gene encodes the highly conserved gamma-aminobutyric acid (GABA) transporter GAT-1, which is responsible for the reuptake of GABA into presynaptic neurons and glia. It is a key regulator for GABAergic signaling in the central nervous system (CNS). A subset of patients presenting with rare forms of infantile encephalopathy with intellectual disability (ID) have been shown to be heterozygous for loss-of-function mutations in SLC6A1. To test our hypothesis that broad delivery of the human SLC6A1 gene across the CNS using adeno-associated virus type 9 (AAV9) to restore normal GABAergic signaling can provide an effective treatment and long-term therapeutic benefit for SLC6A1-Related Disorder, we designed two AAV9 vectors with different promoters (one is a weak universal promoter - JeT; the other is a neuronal-specific promoter - MeP229) and conducted preclinical gene therapy studies using SLC6A1 knockout (KO) mice, a mouse model of SLC6A1, at different developmental ages. The two AAV9/SLC6A1 vectors were tested separately via intracerebroventricular (ICV) administration at neonate age and lumbar intrathecal (IT) administration at the age of postnatal day (PND) 7-10 or 28-35. Neonatal ICV administration of both vectors ($3x10^{11}$ vg per mouse) resulted in significantly reduced seizures assessed by EEG in both SLC6A1 KO and heterozygous mice, as well as improvement in several behavioral tests (fear conditioning, nest building, rotarod performance, and hindlimb clasping) in homozygous KO mice. However, some adverse effects (including death) were noted with both constructs after neonatal ICV treatment. IT administration ($7.5x10^{11}$ or $7.0x10^{11}$ vg for JeT and MeP229 constructs, respectively) to PND7-10 SLC6A1 KO mice showed moderate improvement, but treatment at PND 28-35 had no apparent treatment benefit. The treatment was tolerated well following IT administration at PND 7-10 or PND 28-35 in both KO and WT mice up to one year post-injection. It is not clear whether the stronger efficacy and toxicity seen by neonatal ICV administration is due to the much higher transduction efficiency by this route and age, or whether the earlier intervention was the main driver of efficacy and adverse effects. To increase the transduction efficiency at an older age, the MeP229-SLC6A1 construct was packaged into AAV-PHP.eB and dosed into PND23 mice by intravenous (IV) administration. All the PHP.eB/SLC6A1 treatments ($2x10^{10}$, $2x10^{11}$ or $1x10^{12}$ vg per mouse) still failed to reduce seizures in SLC6A1 KO or heterozygous mice, and the $2x10^{10}$ or $2x10^{11}$ vg doses only showed a moderate nest building behavioral improvement (the 1 $x10^{12}$ vg group wasn't assessed). While AAV-PHP.eB treatment increased the transduction efficiency of SLC6A1 at PND23, no adverse effects were observed across all the IV treated animals. Overall, our data demonstrate compelling efficacy when mice are treated at the newborn age, which is contrasted with a degree of adverse effects specific to this treatment age. Treatment at any older ages (PND7-10, PND23, and PND28) has thus far yielded only modest benefits, although treatment at these ages has lacked any adverse effects. It remains an open question as to which is the key driver of safety and efficacy with this approach: age of intervention or transduction efficiency.

690 An Engineered β-glucocerebrosidase with Improved Stability and Cross-Correction for the Potential Treatment of Gaucher Disease

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Gaucher Disease (GD) is an inherited autosomal recessive lysosomal storage disorder resulting from mutations in the *GBA1* gene that lead to loss of function of the lysosomal enzyme β-glucocerebrosidase (GCase). The resulting accumulation of glucosphingolipids in peripheral macrophages (non-neuronopathic, Type 1) and neuronal cells (neuronopathic, Type 2 acute and Type 3 sub-acute) drives disease pathology in the periphery (splenomegaly, hepatomegaly, thrombocytopenia, and bone infarctions) and central nervous system (neuroinflammation), respectively. Although there are approved enzyme replacement therapies (ERT) for Type 1, there exists a clear unmet need for Types 2 and 3. In addition, the bimonthly burden of ERT infusion, the progression of symptoms despite treatment, and the persistence of bone lesions for some patients warrants development of a gene therapy with durable efficacy. GCase ERT bears a modified glycan structure relative to natively expressed protein, which is required for effective uptake into target cells. Further, wild-type (WT) GCase expresses poorly and is rapidly inactivated in human serum. Our approach to circumvent the intrinsic limitations of GCase protein was to identify GCase variants with exceptional expression efficiency, stability in circulation, and efficient uptake into macrophages. We applied our CodeEvolver® protein engineering platform to screen >5,000 GCase variants over three rounds of iterative directed evolution. The lead GCase variant expresses approximately 200-fold more active protein than WT GCase from Expi293 cells, is more stable in serum, and has increased activity in treated macrophages. Treatment of *GBA1* knockout macrophages with the engineered GCase variant clears glucosphingolipids from the cells efficiently. Finally, we discuss the translational potential for

the evolved *GBA1* transgene delivered to a mouse model of Gaucher disease via AAV. Collectively, our findings highlight the benefit of protein sequence optimization to generate GCase enzyme variants as the next generation of therapies to improve patient outcomes.

691 CNS Gene Therapy Spatially and Temporally Modulates Transcriptional Expression of Neurons and Oligodendrocytes in Canavan Disease

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Gene replacement for neurodegenerative diseases using recombinant adeno-associated viruses (rAAVs) has made great strides in recent years, enabling clinical trials and the first FDA approved gene therapies. Neurodegeneration is progressive and key aspects for clinical intervention include insight into differential responses of gene therapy on brain regions and identifying disease stages that are too advanced for treatment. Using a progressive neurodegenerative disease model, Canavan disease (CD), we characterized region-specific effects of gene replacement on disease reversal and identified treatment stages at which neuropathological recovery is achievable. CD is caused by genetic mutations in aspartoacylase (ASPA), an enzyme found in oligodendrocytes. Defective ASPA affects oligodendrocyte and neuronal homeostasis, triggering progressive loss of both cell types. CNS expression of rAAV-delivered ASPA (rAAV-ASPA) in CD mouse models prevents cellular loss and rescues disease phenotypes. However, brain region response to treatment and the limits of treatment age on therapeutic effect is not known. We hypothesize that disease reversal is age-related and dependent on transcriptomic regulation following region-specific gene correction. Using symptomatic CD mice, we treated juvenile, adult, and mature adult ages with rAAV-ASPA to characterize region-specific transcriptional response of oligodendrocyte and neuronal lineages in an age-dependent manner. Without gene therapy, juvenile CD mice had more PDGFRa+ precursor oligodendrocytes, but significantly reduced pre-myelinating (Olig2+) and mature myelinating (MBP+) oligodendrocytes, two essential cell types required for axonal myelination. Remarkably this phenotypic shift in oligodendrocyte lineage was reversed as early as one-week in juvenile rAAV-ASPA mice and normalized by four-weeks, a finding which paralleled electron microscopy detection of augmented axonal myelination. These effects were largely brain-region dependent; i.e. the corpus callosum, a densely myelinated brain region affected in CD, exhibited significantly elevated PDGFRa+, Olig2+, and MBP+ cells post rAAV-ASPA, suggesting a potentially novel mechanistic effect of remyelination post-gene therapy. Interestingly, this increase of mature oligodendrocytes corresponded with greater expression of mature myelinated (RbFox3+) neurons and neuronal growth cones (Gap43+) in treated mice. Extent of disease progression plays a big role in gene therapy response. We found that adult CD mice with rAAV-ASPA achieved partial restoration of motor function and near-normalized neuropathology. However, therapeutic effect in mature adult CD mice was less pronounced, suggesting late-stage gene-therapy intervention may be difficult to achieve. In conclusion, rAAV-directed ASPA in the CNS can alter the transcriptional regulation of oligodendrocyte and neuronal lineage in a brain-region and age-dependent manner. These changes correlated with observed corrected histopathology, particularly of myelination, in the brain. By leveraging gene therapy in this age-dependent approach, we can better address avenues to treat advanced neurodegeneration in a clinical setting.

692 Determining the Role of CTCF Loops in Neuron Specific Paternal Imprinting of *UBE3A*

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Angelman syndrome (AS) is a neurogenetic disorder caused by loss of *UBE3A*. Expression is biallelic in non-neurons but undergoes paternal imprinting in neurons. An antisense transcript is responsible for this silencing, but it is currently unknown how it is regulated. This transcript originates upstream at *SNRPN*, extends past *IPW* and continues antisense to *UBE3A* (*UBE3A-ATS*), however in non-neurons it terminates at *IPW*. The presence of binding sites for CTCF within this region suggest that it may perform a boundary function. The design strategy for *UBE3A* unsilencing to treat AS is limited by the lack of understanding of how tissue-specific imprinting is controlled at this locus. We have developed an innovative system to study this critical boundary region using epigenetic editing tools and a human neuronal precursor model. Since CpG methylation inhibits CTCF's binding and subsequent looping activity, I hypothesize that CTCF creates a chromatin loop in non-neurons that prevents the extension of *UBE3A-ATS* past *IPW*. Furthermore, CTCF is evicted by DNA hypermethylation in neurons, allowing extension of the antisense transcript past *IPW*. The objective of this research is to characterize the tissue-specific differences in methylation and chromatin structure at *IPW* that determine how *UBE3A* imprinting is controlled by CTCF loops. An immortalized human dopaminergic neuronal precursor cell line (LUHMES) that is not derived from neoplasia presents an epigenome that's closer to wild type. Using the undifferentiated LUHMES as a model for non-neurons and differentiated LUHMES to represent neurons reduces confounding variables and approximates a more direct comparison. Results show that *UBE3A-ATS* is only expressed in neuronally differentiated LUHMES, increases exponentially after day 4 and peaks at day 7 of being in differentiation media. To establish differences in CTCF loops between neuronal precursor cells and mature dopaminergic cells a HiChIP was performed on differentiated and undifferentiated LUHMES cells. The data was analyzed and compared using individual and concatenated samples at 5 kb bins. Four novel CTCF loop interactions unique to neurons were discovered for the Angelman locus. A 4C experiment has also been performed to validate the tissue specific differential looping seen in the HiChIP. In the future we will use dCas9 epigenetic editing to determine if these loops are sufficient and necessary to stop the extension of *UBE3A-ATS* past *IPW.* Use of the LUHMES cell culture model has the potential to accelerate the study of this *UBE3A* boundary region and reveal the role that chromatin loops may play in its regulation of *UBE3A-ATS*.

693 Extracellular Vesicle-Based Delivery of Silencing Sequences for the Treatment of Machado-Joseph Disease | Spinocerebellar Ataxia Type-3

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Machado-Joseph disease (MJD)/Spinocerebellar Ataxia Type 3 (SCA3) is the most common autosomal dominantly inherited ataxia worldwide. It is caused by an overrepetition of the trinucleotide CAG within the ATXN3 gene which confers toxic properties to the ataxin-3 (ATXN3) mRNA and protein. Despite no disease modifying treatment is available up to date, RNA interference technology has shown promising therapeutic outcomes but still lacks an efficient non-invasive delivery method to the brain. Extracellular vesicles (EVs) are cell-derived lipid membranes emerging as a promising delivery strategy due to their capacity to deliver small nucleic acids, such as miRNAs. miRNAs were found to be enriched into EVs due to specific signal motifs designated as ExoMotifs. In this study, we aimed at investigating whether ExoMotifs would promote the packaging of engineered miRNA-based silencing sequences into EVs to be used as therapeutic vehicles to the brain to treat MJD/SCA3 upon daily intranasal administration. We found that miRNA-based silencing sequences, associated with the ExoMotif GGAGGAG and the ribonucleoprotein A2B1 (hnRNPA2B1), retained the capacity to silence mutant ATXN3 (mutATXN3) and were 2.5-fold enriched into EVs.Furthermore, the bioengineered EVs containing the neuronal targeting peptide RVG on the surface significantly decreased mutATXN3 mRNA and protein levels in primary cerebellar neurons from MJD YAC 84.2 and in a novel dual luciferase MJD reporter animal model, upon daily intranasal administration. Altogether, these findings indicate that bioengineered EVs carrying miRNA-based silencing sequences can be used as a promising delivery vehicle for brain therapy.

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694 Gene Editing Therapy in a Humanized *SOD1*^{G93A} Mouse Model of Amyotrophic Lateral Sclerosis

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Background: Amyotrophic lateral sclerosis (ALS) is a rapidly progressing neurodegenerative disease that usually causes respiratory failure and death within two to five years following disease onset. In Europe and the United States, about 2 to 5 out of 100,000 people are suffering from ALS. The estimated number of 2020 ALS cases across the 22 countries is 121,028 prevalent and 41,128 incident cases. About 10% of ALS patients are familial, and most of them are caused by mutations in genes such as *C9orf72, SOD1, TDP-43, and FUS*. Mutations of *SOD1* are associated with ~20% of familial ALS and ultimately induces neuronal death in both the brain and spinal cord. Knockdown of *SOD1* can effectively inhibit the death of motor neurons caused by the accumulation of mutant SOD1; therefore, SOD1 has become an important target for the treatment of ALS. At present, there are ASO and AAV-miRNA drugs targeting *SOD1* in clinical trials phase; however, no effective therapeutic drugs have been approved. **Methods**: To investigate the treatment efficacy of AAV carrying the system of high-fidelity Cas12i (hfCas12Max) and gRNAs targeting *SOD1* (gSOD1), we administrated AAV-hfCas12Max-gSOD1 in neonatal humanized *SOD1*^{G93A} mice (Day 0 to 1) via intrathecal (IT) injection. The indel efficiency and knockdown level of SOD1 protein were tested by PCR plus sequencing and western blot in spinal cord and the whole brain of humanized *SOD1*^{G93A} mice. The lifespan of humanized *SOD1*^{G93A} mice with or without AAV-hfCas12Max-gSOD1 was observed. **Results**: We evaluated the efficiency of *SOD1* knockdown in spinal cord and the whole brain and observed that indel ratio of *SOD1* gene reached 20.3% in lumbar (Fig. 1A). Additionally, the knockdown efficacy of SOD1 protein level was more than 90% in spinal cord of humanized *SOD1*^{G93A} mice at 2 weeks post injection (Fig. 1B), compared with that in control mice. Median survival for low-dose injected humanized *SOD1*^{G93A} mice was 49 days longer compared with humanized *SOD1* ^{G93A} mice with PBS injection (153.8±5.96 days, representing a 32.8% extension of survival (Fig. 1C). Four of the twelve humanized *SOD1*^{G93A} mice treated with AAV-hfCas12Max-gSOD1 survived over 200 days, whereas no humanized *SOD1*^{G93A} mice treated with PBS lived past 161 days. **Conclusions:** Our findings provide a strong foundation and promising approach for the clinical application of AAV-hfCas12Max-gSOD1 to treat ALS patients with *SOD1* mutations. Our gene editing strategy may potentially increase the lifespan and improve motor function of patients with ALS for an impactful treatment in the near future.

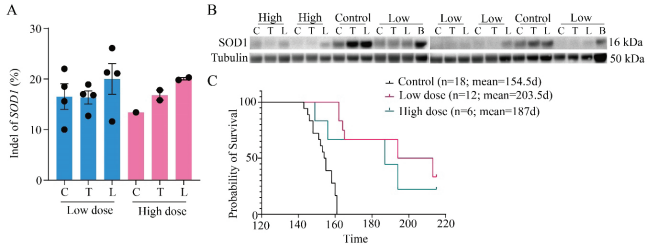

Figure 1. The treatment effect of AAV-hfCas12Max-gSOD1 in the humanized *SOD1*^{G93A} mice

695 Development of AAV-GBA1 Gene Replacement Therapy via Single-IV-Delivery with a Blood Brain Barrier Penetrant AAV Capsid

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Lysosomal enzyme β-glucocerebrosidase (GCase) is encoded by the *GBA1* gene and loss of function (LOF) mutations in GBA1 result in a deficit of GCase activity. This leads to accumulation of glycosphigolipid-substrate such as glycosylsphingosine and glycosylceramide. Bi-allelic GBA1 mutations result in Gaucher disease (GD). Interestingly, heterozygous LOF mutations in GBA1 are also one of the greatest genetic risk factors for Parkinson's disease (PD) and Lewy Body Dementia. Enzyme replacement therapy (ERT) or substrate reduction therapy (SRT) are currently prescribed for GD patients to alleviate clinical impact in the peripheral organs. However, ERT/SRT are often unable to adequately cross the blood-brain barrier (BBB). Adeno-associated viral (AAV)-based gene replacement therapies using a BBB penetrant capsid could provide sustained correction of lysosomal storage disorders affecting the central nervous system (CNS). Successful transduction in areas affected in PD could potentially alleviate GCase activity deficits, reduce glycosphingolipid-substrate accumulation, and improve clearance of alpha-synuclein pathology. Optimized GBA1 transgenes were screened via *in vitro* and *in vivo* assays to demonstrate broad CNS biodistribution and target engagement in WT and GBA1 LOF mouse model. Single-intravenous delivery of top transgene candidates packaged into a mouse BBB-capsid (PHP.eB) were compared in the GBA1 LOF mouse model. Our data show increased biodistribution, expression, and GCase activities, accompanied by reduced neurofilament light chain, and rescue in motor impairment. These data support the testing in non-human primates (NHPs) to select optimal novel BBB-penetrant capsid(s), where we were able to show successful transduction in key areas affected in PD patients.

696 A Novel Small NPC1 Promoter Enhances AAV-Mediated Gene Therapy in Mouse Models of Niemann-Pick Type C Disease

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Introduction: Niemann-Pick Type C Disease (NP-C) is a lethal monogenic neurovisceral lysosomal storage disease. In 95% of cases, it is caused by mutations in the *NPC1* gene that encodes for the lysosomal transmembrane protein, NPC1. There is currently no major disease modifying treatment available. We have previously demonstrated the potential of AAV9-mediated gene therapy as a treatment for NP-C. However, the large size of the *NPC1*cDNA sequence requires a small but efficient promoter to remain within the AAV packaging size limitation. Here, we investigated the ability of a minimal endogenous NPC1 promoter to reduce the size of the expression cassette and drive efficient human *NPC1* gene expression. **Methods:** We designed and investigated the therapeutic efficacy of a novel, minimal NPC1 endogenous promoter sequence compared to other commonly used promoters in gene therapy studies and clinical trials in mouse models of NP-C. Survival, locomotor function and neuropathology were assessed as readouts of efficacy. **Results:** Following neonatal intracerebroventricular injection into the CNS of both Npc1 KO mice and an Npc1 missense point mutant model, our minimal NPC1 promoter provided optimal therapeutic efficacy compared to all other promoters including a significant increase in survival, improved behavioural phenotypes, and attenuated neuropathology. Interestingly, the promoter activity was only optimal on an NPC1 deficient background and not in wildtype cells or wildtype mice, suggesting a specific activity that responds to disease phenotype. **Conclusion:** Our NPC1 minimal promoter is optimal compared to other commonly used promoter sequences in driving human *NPC1* gene expression in mouse models of NP-C. It is also small enough to allow efficient packaging of the AAV genome and suggests an AAV9-based gene therapy has potential for clinical development to enable effective treatment of NP-C. Further clinical translation studies are being undertaken with our industrial partner, Bloomsbury Genetic Therapies Ltd.

697 Engineering Ligand-Gated Ion Channels for Chemogenetic Modulation of Hyperexcitable Circuits in Neurological Disorders

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The fundamental cause of many neurological disorders can be attributed to the abnormal electrical activity of specific neural circuits. Normalizing activity in these dysfunctional neurons should therefore be a valuable avenue for the development of therapeutics, and ultimately the treatment of various nervous system conditions. However, for this to be a successful therapeutic approach, specific targeting is required so that anomalous neuronal activity can be corrected in the dysfunctional circuits without altering the properties of neighboring cells and brain regions. To advance the treatment of neural circuit disorders, Sania Therapeutics is leveraging a combination of intersectional technology platforms that allow for the targeted delivery of chemogenetic proteins through engineered AAV capsids and promoters. This approach will allow us to specifically modify neural circuit activity in disease states while minimizing side effects associated with current therapies. Chemogenetics refers to the alteration of electrical activity in neurons via the use of a genetically delivered protein that responds to a systemically delivered ligand. From a therapeutic perspective, the ideal chemogenetic system would have several properties. i) Minimal effects on baseline neuronal activity without the presence of an activator; ii) Potent and titratable effects on neuronal activity in response to

an activator; iii) validated efficacy in humans.Here we present data showing the efficacy of two chemogenetic cargo candidates for reducing excitability in hIPSC neurons. Using patch clamp electrophysiology, we assessed the effect of cargo expression on baseline neuronal excitability (no activator) and sensitivity to low doses of a specific cargo activator. Our data show that AAV-mediated expression of our lead candidate, SRX-C490, has no effect on baseline firing in hIPSC neurons, but low doses of activator significantly reduced excitability. In contrast, the activator has minimal effect on GFP transfected control neurons. Next, we attempted to increase activator potency using a rational design approach to mutate the genetic cargo, named SRX-C500. SRX-C500 showed increased sensitivity to the activator for threshold (rheobase) and sub-threshold (resting membrane potential) metrics, with larger effect sizes seen at the same doses of activator used with SRX-C490. However, SRX-C500 expression also potently suppressed baseline action potential firing, suggesting additional modifications are needed to reduce the baseline effect whilst maintaining activator sensitivity. Both chemogenetic proteins provide a potent means to develop new therapies based on altering neuronal activity. Due to the lack of effect on baseline excitability and high sensitivity to activator in hIPSC-derived neurons, SRX-C490 is now being tested as an intramuscular delivered, AAV treatment for motor neuron hyperexictability in an in vivo mouse model of spasticity.

698 Low-Dose Intracerebroventricular Delivery of a Second-Generation AAV Gene Therapy for Spinal Muscular Atrophy Achieves Efficient and Toxicity-Free Motor Function Rescue in Mice

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Onasemnogene abeparvovec (brand name Zolgensma®) is an FDA-approved adeno-associated virus (AAV) vector gene therapy that has been a significant breakthrough for treating infants with SMA. The vector produces ubiquitous expression of the human survival motor neuron 1 transgene under the cytomegalovirus enhancer/chicken β-actin promoter (*CMVen/CB-hSMN1*). However, the high intravenous (IV) dose required for efficacy (1.1E+14 vg/kg) can cause systemic adverse events, most commonly gastrointestinal (elevated liver transaminases and vomiting), but also hematologic (thrombocytopenia, thrombotic microangiopathy, increased prothrombin time) and cardiac (elevated troponin I). The US prescribing label for onasemnogene abeparvovec includes a black box warning of acute liver injury and acute liver failure. To improve upon the safety and efficacy of gene therapy for SMA, we developed a novel second-generation (2nd-gen) *hSMN1*-AAV gene therapy vector consisting of the endogenous *SMN1* promoter and a codon-optimized *hSMN1* transgene. In a previous head-to-head comparison, our 2nd-gen vector showed therapeutic advantages over a "benchmark" vector, whose design is identical to onasemnogene abeparvovec when administered intravenously to SMA mice, including longer life span, absence of liver toxicity, and better restoration of muscle function. To identify the capsid and route of administration that provides the best delivery to brain and spinal cord, we tested the 2nd-gen and benchmark constructs packaged into AAV9 or AAVrh10 capsids and delivered by intracerebroventricular (ICV) injection into SMA mice at P0. We compared the therapeutic outcomes of the four vectors at 3.3E+13 vg/kg (high dose) or 1.1E+13 vg/kg (low dose). At the high-dose, the AAV9-2nd-gen vector outperformed the other three vectors in survival, weight gain, righting reflex and the rotarod test, whereas the AAVrh10-2nd-gen vector performed the best in the grid test. At the low dose, the AAV9-2nd-gen vector also performed the best, followed by the AAVrh10-2nd-gen, AAV9-benchmark, and AAVrh10-benchmark vectors. We conclude that the 2nd-gen vector provides better efficacy than the benchmark vector following ICV delivery in both AAV9 and AAVrh10 serotypes. Western blot analysis demonstrated higher SMN1 protein expression in the brain and spinal cord of SMA mice treated with the 2nd-gen vector than with the benchmark vector, irrespective of the capsid used. Abundant SMN1 protein expression occurred in the dorsal root ganglia (DRG) of SMA mice in all treatment groups, but no clear evidence of DRG-related toxicity was observed during the 90-day study period. We injected the AAV9-2nd-gen vector into wild type mice at a dose of 1.0E+14 vg/kg by ICV and found no abnormalities associated with DRG toxicity for up to one year. Intravenous delivery of AAV9-2nd-gen at 3.3E+14 vg/kg and ICV delivery at one-tenth the dose (3.3E+13 vg/kg) produced similar rescue of lifespan and body weight gain. Importantly, ICV delivery achieved better motor function improvement than IV delivery, as determined by the righting reflex, grid test and rotarod test. Conversely, IV delivery was more efficient in reducing SMA disease-related complications observed in peripheral tissues, such as tissue necrosis of the ear, eye, and tail. In summary, we have engineered novel AAV based 2nd-gen vectors for SMA gene therapy with transgene expression that better mimics the physiological levels of SMN1 in transduced tissues. When delivered by ICV administration, the AAV based 2nd-gen vectors have the potential to be a safer, more effective, and more efficient clinical approach for treating SMA.* **G.G., and J.X are co-corresponding authors**

699 Characterization of In-Vivo Models of the Pediatric Neurodegenerative Disorder BPAN

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Beta-Propeller Protein-Associated Neurodegeneration (BPAN) is a rare neurologic disease associated with early developmental delays and progressive neurodegeneration with movement disorder starting in adolescence. This X-linked disease is caused by mutations in the WDR45 gene encoding WIPI4, a WD40 repeat-containing PtdIns(3)P binding protein involved in autophagosome formation. There is currently no therapy for this disease, and little is known about the molecular mechanisms behind its pathological manifestations. The goal of this study is to test whether AAV-mediated WDR45 gene expression can rescue disease phenotype in BPAN mouse and cell models. To achieve this goal, we first set out to characterize BPAN mouse and cell models and establish disease-relevant outcome measures. BPAN

mouse model, *WDR45* $^{c.ex9+g>a/Y}$ mice, were obtained from the national public repository system for mutant mice (MMRRC) and mimic the human variant c.830+1G>A documented in a BPAN patient. The mice were successfully bred to establish an in-house colony, and their phenotype has been evaluated using a battery of tests. In the open field test, starting at two months of age *WDR45* $^{c.ex9+g>a/Y}$ mice exhibit increased track length, ambulatory events and walking time compared to litter-, sex- and age-matched WT controls. This trend persists in older naïve mice tested in the open field test at 11.5 months of age. The observation of increased ambulatory activity is indicative of hyperactivity in BPAN mice and is consistent with clinical observations of attention deficits and hyperactivity in BPAN children, and therefore can be a disease-relevant outcome measure for assessing preclinical efficacy in this model. Starting at 2 months, mice were tested monthly in the rotarod apparatus, until they reached 11 months of age. No difference was detected in the younger animals, while at 11 months *WDR45* $^{c.ex9+g>a/Y}$ mice fall of the rod sooner compared to controls, suggesting a motor function, balance and coordination deficits later in the course of disease. However, worsening of the rotarod performance was paralleled by weight gain in *WDR45* $^{c.ex9+g>a/Y}$ mice that become significant starting at 9 months of age. Weight gain in *WDR45* $^{c.ex9+g>a/Y}$ mice may be a confounding factor for rotarod performance in mice, and therefore evaluation of motor function, balance and coordination warrants further investigation. In addition to behavioral tests, we used a "neuroexam" panel of short tests to evaluate overall neurologic health, including righting reflex, crossed extensor reflex, forelimb and hindlimb placing responses, grasp, postural, rooting reflexes, placing, visual placing, eye blink and acoustic startle responses and negative geotaxis. Neuroexam showed no significant differences between *WDR45* $^{c.ex9+g>a/Y}$ and *WDR45* $^{+/Y}$ mice, suggesting intact basic sensory function. Our histopathological analysis of post-mortem brain tissue revealed astrogliosis and microgliosis in the cortex of 3-month-old *WDR45* $^{c.ex9+g>a/Y}$ mice, and in the striatum at 6 months of age. Evaluation of histopathological hallmarks of neuronal health/neurodegeneration is currently in progress. Overall, our data show that *WDR45* $^{c.ex9+g>a/Y}$ mouse model mimic major manifestations of the human disease, and is a great tool for BPAN gene therapy efficacy testing.

700 AAV-Mediated Gene Therapy to Target the Progressive Neurodegeneration in a Mouse Model of Xeroderma Pigmentosum - Cockayne Syndrome (XP-CS)

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The process of DNA repair is critical for the maintenance of genome integrity and stability within cells throughout an organism. Pathogenic variants in genes expressing proteins involved in DNA repair mechanisms can lead to serious multi-organ dysfunction. Xeroderma pigmentosum (XP) and Cockayne Syndrome (CS) are rare autosomal recessive disorders arising from deficiencies in proteins involved in the nucleotide excision repair (NER) pathway, a key component of DNA repair. Patients with XP and CS have no disease altering therapy available, beyond supportive care. Xeroderma pigmentosum complementation group G (XPG) is a DNA endonuclease responsible for the 3' incision made during NER. XPG is encoded by the *ERCC5* gene and pathogenic variants in *ERCC5* result in a disorder with characteristic skin photosensitivity. Approximately 30% of XP patients develop a more severe form of the disease that includes accelerated aging, neurodegeneration and other symptoms similar to those observed in CS patients including microcephaly, growth defects, hearing loss, and cognitive disability. This more severe form of the disease is referred to as XP-CS. The goals of this study were to characterize XP-CS related physiological and neurological outcome measures in an XPG mouse model to provide a platform for assessing the safety and efficacy of adeno associated virus (AAV)-mediated gene therapies, and then to test a candidate AAV gene therapy in this model. Age matched Xpg-/- and wild-type (WT) sibling control mice were evaluated for the following over a 12-week period: body length, weight, ataxia, tremors, and kyphosis. Xpg-/- mice showed significant growth defects from birth as compared to sibling controls. By 10 weeks of age all Xpg-/- mice had developed severe ataxia, tremors, and kyphosis. The severity of neurodegeneration as evaluated through weekly hind limb clasping scores, showed a significant increase in score for Xpg-/- mice as compared to WT controls. Further assessments included locomotor activity tracking (ActiTrack) and indirect calorimetry. Xpg-/- mice were hypokinetic, travelled significantly shorter distances, and displayed a reduced metabolic rate compared to WT mice. Tandem mass tagging (TMT) based global proteomic analyses of Xpg-/- mouse brains identified a total pf 2748 proteins, out of which 198 showed significantly altered levels ($p<0.01$). An *AAV9-ERCC5* gene therapy vector was administered to 6-week-old Xpg-/- mice through intrathecal (IT) injections at a dose of $1x10^{12}$ vg/kg. This treatment strategy delayed the onset of disease symptoms and led to a longer lifespan in treated mice as compared to untreated Xpg-/- controls. Full tissue evaluations are underway. In parallel, continued refinement of the physiological and neurological outcome measures in this animal model and further preclinical testing of our gene therapy strategies will promote further development of this approach towards future human clinical trials for this group of DNA repair disorders.

701 Engineered Human Blood-Brain Barrier Microfluidic Model for Assessing Permeability of Cell and Gene Therapies into the CNS

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Efficient delivery of cell and gene therapies following noninvasive delivery to the central nervous system (CNS) remains a formidable challenge posed by various anatomical and physiological barriers within CNS, most notably, the blood-brain barrier (BBB). Due to

challenges in translating results from animal models to the clinic, relevant *in vitro* human BBB models are needed to assess permeability and enable design and selection of BBB-penetrant therapies for neurological disorders. To address this, we are developing an *in vitro* microfluidic human BBB model for screening and evaluation of therapeutics targeting the CNS. Microfluidic 3D co-culture models can mimic the physiological microenvironment of the BBB and address discordant performance of drugs observed between species. Moreover, implementation of this platform during early stages of drug discovery can accelerate the development of therapies. Thus, our *in vitro* BBB model is a promising tool to study transport mechanisms and aid in the development of cell and gene therapies with increased brain penetrance.

702 A Novel Gene Therapy Approach for ALS by Overexpressing the Pleiotropic Chronokine α-Klotho

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In amyotrophic lateral sclerosis (ALS), muscle denervation and degeneration of motoneurons result in progressive muscle weakness and atrophy. Motor impairment leads to loss of autonomy, need for intensive care and early death of the patients, without effective treatments being available. Preventing axonal detachment from muscles, protecting motoneurons and promoting reinnervation are key to improve the functional outcome of ALS. For preserving neuromuscular function in the $SOD1^{G93A}$ mouse model, we synergically influenced on motoneuron terminals and muscles by boosting the secretion of α-Klotho in skeletal muscles. α-Klotho is a pleiotropic chronokine with an excellent profile as neuroprotective and myoregenerative agent by means of anti-oxidative and anti-inflammatory properties, promoting myelination, protecting from excitotoxicity, and maintaining mitochondrial ultrastructure and function. To overexpress α-Klotho in the skeletal muscles of $SOD1^{G93A}$ mice, AAV8 vectors were systemically administered at a dose of $3x10^{14}$ vg/kg at an early stage of the disease. Secretion of α-Klotho by muscles enhanced motor function and strength of the animals and delayed the onset of the disease. Neuromuscular functional improvement was reflected as increased compound muscle action potential (CMAP) amplitudes and by larger size and number of functional motor units of hindlimb muscles compared to mock-treated controls. α-Klotho-treated $SOD1^{G93A}$ mice showed more surviving motoneurons and a significant reduction in microglial and astroglial reactivity in the ventral horn of the spinal cord. A greater amplitude of the motor evoked potentials (MEPs) also evidenced improved connections between corticospinal and spinal motoneurons. At the neuromuscular level, there was a higher number of occupied motor endplates and a preserved mass of the muscles. In view of the high doses of AAV8 needed to reach therapeutic efficacy, we moved to a myotropic AAV vector. With a 20-fold decrease in the dose, we achieved higher preservation of neuromuscular connectivity, motor performance and strength, and delayed the disease onset in $SOD1^{G93A}$ mice, even further than with the AAV8-α-Klotho treatment. More importantly, when $SOD1^{G93A}$ mice were treated at 11 weeks of age, when already symptomatic, muscle overexpression of α-Klotho slowed down the progressive decline of the neuromuscular and motor function characteristic of $SOD1^{G93A}$ mice. Overall, our results provide evidence that the overexpression and secretion of α-Klotho in muscles can promote functional improvement in ALS and may open a new avenue for the treatment of this devastating disorder.

703 Development of an AAV Gene Therapy for Hereditary Spastic Paraplegia Type 4 (SPG4-HSP)

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Hereditary spastic paraplegia type 4 (SPG4-HSP) is an inherited neurodegenerative disorder and is the most common form of hereditary spastic paraplegia (HSP). SPG4-HSP is an autosomal dominant disease caused by mutations in the SPAST gene that encodes the microtubule severing enzyme spastin. It is considered that SPAST mutations not only disrupt the physiological functions of spastin, but elicit toxic gain-of-functions by the mutated gene products. Different mutations in the SPAST gene have been described, however the genotype-phenotype correlation remains unclear, even among affected members of the same families. SPG4-HSP has been classified in two major forms, termed pure and complex. Pure SPG4-HSP is the most common and is characterized by progressive spasticity of the legs, leading to stiffness and gait defects. Symptoms of complex SPG4-HSP include muscle weakness in the legs but can also include loss of sensation in the feet, and cognitive impairment among others. The age of symptom onset is variable, but most commonly begins in early adulthood. We have developed several dual function AAV vectors encoding wild-type human spastin and a micro RNA (miRNA) to silence the endogenous alleles. We delivered these AAV vectors into wild-type mice (n=10) by neonatal intracerebroventricular injection (4E10 vg/mouse) at postnatal day 1 and sacrificed them at 4 weeks of age. We are assessing spastin expression, silencing of the endogenous spastin mRNA, and biodistribution in the CNS and peripheral organs using western blot and qPCR. Our team is also conducting efficacy

studies in an SPG4 mouse model using a first generation AAV-SPG4 vector. A similar disease has been described in Brown Swiss cattle, also caused by a mutation in the SPAST gene, called Bovine Spinal Dysmyelination (BSD), more commonly known amongst farmers as Spinal Dysmyelination (SDM). Notably, the bovine disease presents a more severe phenotype than humans and affected calves are unable to stand at birth. We have obtained cattle with BSD to use as a large animal model of SPG4 and further test the efficacy of our vectors. Altogether, these combined studies in small and large animal models will help develop a treatment for patients with SPG4-HSP.

704 High-Throughput Quantitative Neurohistology for Gene Therapy Vector Evaluation

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In order to effectively treat disease, in vivo gene therapy vectors need to restore normal expression levels of the disease-relevant protein. This requires not only that the vector cross the blood brain barrier, but also that it deliver the payload to the appropriate regions of the brain, and the appropriate cell types. Furthermore the correct level of expression must be acheived in a sufficient portion of the targeted cells. Among all the preclinical methods commonly used to measure gene therapy payload delivery , neurohistology is uniquely positioned to illuminate all of the questions above. However, neurohistology as typically practiced is neither quantitative or capable of high throughput data generation and analysis. Expressive Neuroscience has created a platform that reenvisions histology for gene therapy discovery. Using streamlined lab protocols in conjunction with machine-learning assisted informatics we are able to automate measurement of target protein expression in thousands of cells in a typical experiment. Here we show, in the context of a rodent Rett syndrome model, how our platform can be used to quantitatively map payload expression across the brain at a cellular scale to support gene therapy discovery efforts.

705 Increased CNS Transduction is Realized Following Peripheral Injection in Animals Suffering from Experimental Autoimmune Encephalomyelitis

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Multiple Sclerosis (MS) is the prototypical neuro-inflammatory demyelinating disease. Within MS, the development focal lesions is considered a hallmark and leads to axonal damage, axonal death, and progressive disability in late disease stages. Current MS therapies supress the inflammatory response within the CNS, only a portion of disease. Currently, there are no FDA approved treatments for combating demyelination.Remyelination is the regenerative process where new myelin sheaths are formed and axonal function is restored. Within MS, remyelination occurs but is inefficient and ultimately fails in the majority of lesions. Thus, induction of remyelination has been postulated as an effective treatment for MS. This is supported by the fact that patients with a higher capacity to remyelinate show lower levels of disability. However, many obstacles impede the development of effective therapeutics to induce remyelination. Two such obstacles include: (i) crossing the blood brain barrier (BBB), (ii) delivering therapeutics specifically to the site where they are needed, the demyelinating lesion. Adeno-associated virus (AAV) is one of the most widely used vector platforms. Some AAV serotypes are capable of crossing the BBB. Of these, AAV9 is the leading serotype for targeting the CNS. Importantly, AAV9 efficiently transduces astrocytes, a major cellular component of demyelinating lesions. Thus, the AAV platform represents a novel treatment modality capable of delivering therapeutics to lesions to induce remyelination. However, AAV9 is inefficient at crossing the BBB when injected i.v. Instead, direct CNS injections are often required for sufficient transduction. These techniques are invasive in nature and have poor CNS bio distribution. An ideal technique would be one that was minimally invasive, resulted in high CNS transduction levels, and achieved excellent biodistribution throughout the CNS.In MS, and its animal model experimental autoimmune encephalomyelitis (EAE), perturbations in the BBB occur allowing entrance of immune cells into the CNS. We hypothesized that this could be leveraged by AAV to gain access to the CNS resulting in improved expression and transduction. In previous work, we showed increased transgene expression in EAE animals though it was unclear if this was due to improved AAV transduction or a result of neuronal inflammation present in our model. Here we show that improved CNS transduction is realized in animals suffering from EAE following a peripheral injection (Fig 1). In the brain, ~6 fold increased transduction was seen in EAE animals as compared to non-EAE controls that received AAV (Fig 1A). In the spinal cord, the primarily affected CNS region in EAE, a ~56 fold increase was seen in EAE animals as compared to controls (Fig 1B). Further, excellent biodistribution of transgene expression resulted with expression being seen in all regions of the brain (Fig 1C) and spinal cord (Fig 1D). Thus, peripherally injected AAV holds promise as a therapeutic for the induction of remyelination in multiple sclerosis.

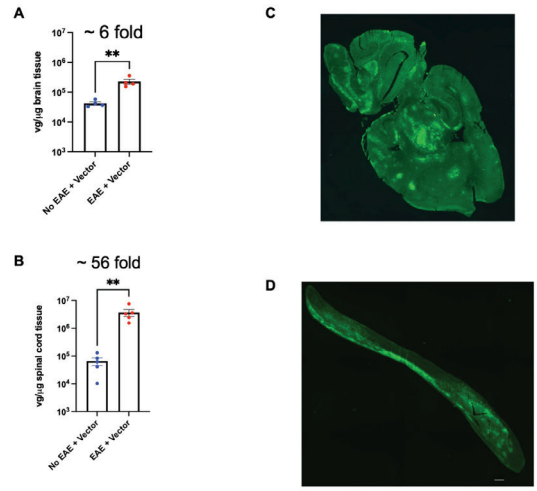

Figure 1: Transduction was evaluated in the CNS of animals following a single peripheral injection. In the brain, a ~6 fold increase in transduction occurred in EAE animals as compared to controls (**A**) In the spinal cord, a ~56 fold increase was seen in EAE animals as compared to controls (**B**). Transgene (eGFP) was also seen in all regions of the brain (**C**) and spinal cord (**D**) showing excellent biodistribution of our vector throughout the CNS. Green is eGFP expression under the control of a truncated GFAP promoter, images taken at 2x, scale bar = 500 μm.

706 AAV Gene Therapy for *GNAO1* Disorders

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Introduction: Mutations in the *GNAO1* gene cause severe neurologic disability including epilepsy and motor dysfunction. The *GNAO1* gene encodes for the $G\alpha_o$ subunit of a heterotrimeric G protein, comprising two isoforms. The pathophysiology related to this monogenetic defect is not fully understood and can be related to gain of function or loss of function. Here we will use a heterozygous knock-in GNAO mouse model ($GNAO^{+/R209H}$) that is genetically similar to humans, and test the therapeutic effect associated with either increasing, or silencing and replacing *GNAO1*. Firstly, we will utilize AAV to supplement GNAO1 isoforms. Alternatively, we will use a dual function AAV vector encoding artificial miRNA to silence endogenous mutated *GNAO1* and express the normal gene. Our goal is to simultaneously understand disease origin and create treatment modalities. **Methods:** We built two monocistronic AAV vectors, each encoding a GNAO1 isoform (GNAO1.1 and GNAO1.2) driven by a CBA promoter. In parallel, we designed a bicistronic AAV vector encoding both GNAO1 isoforms from a centrally located bidirectional promoter. All vectors were transiently transfected into human HEK293T cells, followed by western blot analysis of GNAO1, HA-tag or myc-tag. We then analyzed AAV-derived GNAO1.1 and GNAO1.2 expression levels in transfected cells by qRT-PCR using isoform-specific Taqman assays. To move these experiments *in vivo*, we utilized the $GNAO^{+/R209H}$ mouse model to first study its natural history and then understand treatment efficacy and toxicity. We tested the AAV-GNAO1 vectors by neonatal ICV injection in our mouse model, as well as in WT animals for toxicity assessment. Open field test was utilized to assess phenotypic differences between AAV injected mice and PBS controls. **Results**: Western blot analysis from *in vitro* transfection of monocistronic (scAAV9-GNAO1.1 and scAAV9-GNAO1.2) and bicistronic (AAV9-GNAO1.1/1.2) vectors demonstrated expression of respective GNAO isoforms and qRT-qPCR data revealed appropriate mRNA from AAV-derived GNAO protein i*n vitro.* However, behavioral tests, namely open field test, showed no phenotypic differences between treated and untreated mice. Whereas a comparison study between WT and Hz mice had shown a significantly increased hyperactive state in the Hz mice. There was no evidence of vector toxicity. We are now working on our second approach using the dual vector strategy, and we have selected candidate miR sequences to silence endogenous GNAO1 mRNAs. **Conclusions**:Our data shows that all GNAO1 vectors are expressed in HEK293T cells, as evidenced by protein expression of GNAO, and by qRT-PCR. In our natural history study, we found that heterozygous $GNAO1^{+/R209H}$ mice have increased hyperactivity on the open field behavioral test. Our hypothesis is that altered signaling in the striatum of heterozygous $GNAO1^{+/R209H}$ mice is the major driver of their hyperactivity phenotype. Although our initial treatment study analytics showed no difference in mouse behavior, increasing vector dose may have an effect, and therefore we are currently enrolling more animals in a higher dose studyThis study also provides the most efficient miR^{GNAO1} which we will use to create dual function AAV vectors capable of silencing the endogenous GNAO1 mRNA and expressing normal $G\alpha_o$ protein. Therefore, paving the way for a potential treatment for this devastating disease.

707 Development of an Optimized AAV Gene Therapy Treatment for XLRP Caused by RPGR

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Introduction: Retinitis pigmentosa (RP) is one of the most severe forms of retinal degeneration, which affects 1 in 3000-3500 individuals. X-linked retinitis pigmentosa (XLRP) is generally early onset and severe form of RP, 70% of XLRP cases are caused by mutation in gene encoding retinitis pigmentosa GTPase regulator (RPGR). Half of patients with mutations in RPGR gene become legally blind at 45 years old yet there is still no effective treatment. The retinal-specific isoform RPGRORF15 localized at photoreceptor connecting cilium involving in modulating cargo trafficking, loss of this protein affects phototransduction proteins in the outer segments (OS). Here we utilized a photoreceptor-targeted gene therapy to restore RPGRORF15 expression and inhibit retinal degeneration with subretinal injection in murine model. **Methods:** The human *RPGRORF15* cDNA was codon-optimized and constructed into pAAV2 vector and driven by a photoreceptor specific promoter hRK (human rhodopsin kinase) and packaged in AAV5 serotype. Dose-dependent expression of RPGRO RF15 was examined in 661W cells and in murine retina by western blot. In vivo cellular localization of human RPGRORF15 at mouse photoreceptor was examined by immunofluorescence with human RPGR specific antibody. The safety study was performed in both C57/BL6 mice and New Zealand White (NZW) rabbits at 1 month and 2 months after subretinal injection of AAV5-hRK-*RPGRORF15* respectively. The function of AAV5-hRK-*RPGRORF15* was tested in RPGR knockout mice. **Results:** RPGRORF15 is highly unstable and prone to lose fragments due to its guanine-adenine rich 3' terminal, we developed and optimized *hRPGRORF15* cDNA with high stability without truncation. Dose-dependent expression of hRPGRORF15 in 661W cells after AAV5-hRK-*hRPGRORF15* transduction was confirmed by western blot, similarly in *in vivo* mouse retina after subretinal injection. The cellular localization of human RPGRORF15 was detected in mouse photoreceptor outer segments by immunofluorescence. Safety study in both C57/BL6 mouse and NZW rabbit show subretinal injection of rAAV-RPGRORF15 was well tolerated *in vivo*, no abnormal retinal structure and inflammatory infiltration were observed. RPGR knockout mice showed gradual thinning of the outer nuclear layer, whereas AAV5-mediated RPGRORF15 expression can alleviate the phenotype. **Conclusion:** Our data provides proof-of-concept to the use of AAV ocular gene therapy for the treatment of XLRP caused by RPGR mutation.

708 Discovery and Characterization of a Safe and Efficacious Gene Replacement Therapy for Treating Bietti Crystalline Dystrophy

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Bietti Crystalline Dystrophy (BCD, MIM 210370) is an autosomal recessive inherited disease, which was named for its distinguishing yellow-white crystalline deposits observed in patient's fundus. BCD is one of the most prevalent degenerative retinal diseases in East Asia, especially in China, where the frequency is estimated to be 1 in 20,000. *CYP4V2* has been identified as the disease-causing gene of BCD. *CYP4V2* loss-of-function mutation causes a dysregulation of lipid metabolism. BCD patients are characterized by vision loss and night blindness between 20 and 40 years of age and eventual progression to legal blindness between the ages of 50 and 60. Currently, there is no treatment available for BCD. Our laboratory has generated a knockout mouse model of *Cyp4v3*, the homolog of human *CYP4V2* gene. Changes in the ERG a- and b-waves in the *Cyp4v3*$^{-/-}$ mice were observed after 24 weeks of age, with retinal lesions in the fundi of the mice exhibiting ERG changes. The phenotypes of the *Cyp4v3*$^{-/-}$ mice are milder than those of the BCD patients but consistent with the slow natural course of this degenerative eye disease. To evaluate whether *CYP4V2* gene replacement therapy could be beneficial to BCD patients, we generated and evaluated a recombinant scAAV8 to deliver sub-retinally a human *CYP4V2* gene expression cassette. Its efficacy was demonstrated in the *Cyp4v3*$^{-/-}$ mice and published by our group previously (Qu et al., Gene Therapy, 2020, 27(7-8): 370-382). Recently, an improved *CYP4V2* expression cassette was constructed by codon-optimization and promoter screening to generate a *CYP4V2* transgene recombinant AAV named as VGR-R01, which showed significantly increased expression of the CYP4V2 protein in comparison to the previously published construct**.** Pharmacology and toxicology studies were conducted to evaluate the *in vitro* and *in vivo* efficacy, pharmacokinetic, and safety of VGR-R01. *In vitro* cell-based studies showed that VGR-R01 mediated the expression of functional CYP4V2 protein in cell lines and *CYP4V2*$^{-/-}$ iPSC-RPE cells. An *in vivo* proof-of-concept efficacy study was conducted in the *Cyp4v3*$^{-/-}$ mice, and the results showed that ERG amplitudes of both eyes were significantly improved following a single subretinal injection of VGR-R01 in the unilateral eye at a dose of 4.0×10^{7} vg/eye. A safety pharmacology assessment was incorporated into a 13-week GLP-compliant toxicology study in Cynomogous monkeys, and the results showed that VGR-R01 had no effects on cardiovascular or respiratory systems. In the 13-week GLP-compliant toxicology studies in both New Zealand Rabbits and Cynomolgus monkeys, after a single subretinal injection, VGR-R01 was well tolerated in both species and the MTD (maximum tolerate dose) was 1.6×10^{11} and 2.4×10^{12} vg/eye, for rabbits and monkeys (the highest evaluated dose in each species), respectively. No test article related systemic toxicities or T cell immune responses to the capsid or transgene product were observed. Both species developed antibodies to the capsid, but not to the transgene product. Ophthalmic examination and ocular histopathology findings were most likely resulted from the surgery procedure from the subretinal drug adminstration. Following a single administration, VGR-R01 mainly distributed in the ocular tissues, with no obvious changes in the pattern of distribution in the retina over time. In summary, VGR-R01, a safe and efficacious AAV-based gene therapy for treating BCD has been developed. Encouraging results from preliminary investigator-initiated-trials (IITs) have been obtained with Phase I/II trial patient enrollments ongoing in China.

709 A Bi-Cistronic AAV Vector Aimed at Addressing the Complex Pathology Associated with Geographic Atrophy

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PurposeStrategies that target components of the complement cascade display variable degrees of success in reducing GA growth and are associated with varying degrees of neovascularisation pathology. Metabolic stress to retinal pigment epithelial (RPE) cells, acting through the NLRP3 inflammasome, is hypothesised to play a pivotal role in this disease. We have developed a bi-cistronic viral vector, IKC159V, capable of enhancing expression of membrane co-factor protein (MCP/CD46) and pigment epithelium-derived factor (PEDF) which are both reduced in individuals with GA. As PEDF has anti-angiogenic, retinoprotective, and anti-inflammatory properties, loss of this protein reduces the ability of the RPE to withstand oxidative stress. Loss of endogenous CD46 leaves the retina highly susceptible to complement attack. We report on the *in vivo* efficacy of IKC159V which aims to provide greater efficacy against GA than sole anti-complement approaches. **Methods**IKC159V was evaluated in multiple rodent GA models. Transgene protein expression levels were examined by retinal immunohistochemistry and vitreal ELISA or Western blot after intravitreal or subretinal delivery. Efficacy was evaluated via fluorescein angiography (FA) and choroidal flat-mount analysis 7 days post laser CNV. Functional activity of soluble CD46 (sCD46) was determined through vitreal C3b cleavage assays and PEDF retino-protection was demonstrated 7-days after intravitreal NMDA induced toxicity or 2-days after intravenous sodium iodate (SI) treatment. **Results**Functional activity of sCD46 expressed from the IKC159V vector resulted in significantly more cleavage of C3b to iC3b (IKC159V = 47%, Null = 8% P<0.001) and was as efficacious as a vector expressing CFI only (CFI = 59%). The SI model specifically causes oxidative damage and degeneration of RPE and PRs that model GA pathology. IKC159V was able to show significant RPE sparing following systemic SI measured 2 days later (IKC159V = 44% RPE loss, Null = 71%, p<0.001). In the laser CNV model, IKC159V reduced the proportion of leaky lesions compared to Null vector (IKC159V = 52%, Null = 76%, P<0.001) and attenuated the neovascular area (IKC159V = 8782 ± 1870 pixels, Null = 17403 ± 4520 pixels; P = 0.08). Finally, elevated PEDF levels correlated with retinal ganglion cell neuroprotection from 30nmol NMDA insult (IKC159V = 780 cells, Null = 358 cells P<0.001).**Conclusion**IKC159V leads to expression of functional sCD46 and PEDF that show a reduction in the activation of the complement cascade and protection of retinal cells respectively. Our data suggests that expression of PEDF in combination with sCD46 may slow GA progression and reduce VEGF-induced leakage in addition to neovascularisation. These data support the therapeutic potential of IKC159V for the treatment of GA.

710 Intraocular and Systemic Gene Therapy for Gyrate Atrophy of the Choroid and Retina

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Gyrate atrophy of the choroid and retina (GACR) is due to ornithine aminotransferase (*OAT*) deficiency which causes hyperornithinaemia leading to retinal pigment epithelium (RPE) toxicity and impaired vision. Adeno-associated viral (AAV) vector-mediated *OAT* liver gene transfer lowers ornithinaemia in the *Oat*$^{-/-}$ mouse model of GACR and protects retinal structure and function but without full normalization. We investigated whether restoration of retinal OAT expression improves the retinal phenotype of *Oat*$^{-/-}$ mice. A single subretinal administration of an AAV vector encoding human OAT (AAV-*OAT*) results in intraocular OAT expression, reduced RPE abnormalities and improved thickness of the outer nuclear layer in *Oat*$^{-/-}$ mice up to 12 months of age. However, retinal electrical activity remained reduced, suggesting that hyperornithinaemia, which is not decreased by subretinal AAV delivery, impairs retinal function. We are currently testing if the combination of retinal and liver Oat gene transfer provides higher therapeutic benefit than each of the two separate approaches.

711 Engineered Melanopsin Mutants for Optogenetic Vision Restoration

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There are limited treatment options for blindness caused by the irreversible death of photoreceptors. Without photosensitive cells, the retina cannot send light information to the brain. One strategy to restore light perception in these patients is through optogenetics, where DNA for a light sensitive protein is delivered to the remaining retinal cells, essentially making new pseudo-photoreceptors. Visual resolution is proportional to the number of pseudo-photoreceptors created, so an AAV capable of achieving pan retinal expression through intravitreal injection, like AAV2.7m8, is potentially most advantageous. Previous work in optogenetic vision restoration has demonstrated that both light sensitive ion channels (channelrhodopsins like ChR2 and ChrimsonR) and G-protein-coupled receptor (GPCR) opsins like rhodopsin and the cone opsins have strengths and weakness, and as such, neither are ideal as light sensors. For example, cells expressing channelrhodopsins are much less light sensitive than those with rhodopsin or the cone opsins. These channelrhdopsins are so light insensitive that medical devices such as light amplifying goggles and implantable LED arrays are often required to generate sufficient light for a visual response. Besides being sensitive to ambient light, GPCR opsins can also adapt to light making their overall range of light sensitivity much greater than that of their ion channel counterparts. However, rhodopsin and the cone opsins, when expressed outside of photoreceptors, are extremely slow at responding to the removal of light, reducing visual acuity. In contrast, the channelrhodopsins respond more quickly to light withdrawal and can also more easily source and regenerate their own light sensitive chemical, retinal. Rhodopsin and the cone opsins rely on specific processes supplied by retinal pigmented epithelial (RPE) cells to regenerate retinal that are difficult to impossible to recapitulate when expressed outside of non-photoreceptor cells. The inability to source or regenerate retinal could impair the ability to respond to light over time. Potentially a better light sensor candidate to generate pseudo-photoreceptors for optogenetic vision restoration is melanopsin, the light sensor for circadian rhythm and pupil dilation, which has both the light sensitivity of rhodopsin and the cone opsins as well as the ability to source and regenerate retinal. However, its extremely slow kinetics prevents it from being the ideal light sensor for optogenetic vision restoration. To improve the kinetics of melanopsin, close to one hundred mutants were developed and screened through *in vitro* fluorescent calcium imaging. Mutations to phosphorylation sites, particularly on the c-terminus (CTD), which becomes more likely to bind to signal terminating beta arrestins after phosphorylation, showed improved kinetics over wildtype. Other mutational strategies targeting signal termination and beta arrestin binding through truncation of the CTD as well as substituting it for the CTD of other GPCRs also yielded mutants with faster light response termination. The improved kinetics in these engineered melanopsin mutants suggests they could be utilized as a therapeutic transgene for optogenetic vision restoration.

712 Compact CRISPR/Cas12-Based Mutation Independent Therapy Strategy for Rhodopsin-Associated Autosomal Dominant Retinitis Pigmentosa via Single AAVs

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Background: Retinitis Pigmentosa (RP), a group of inherited, retinal degenerative diseases resulting in vision loss and blindness, is the most common inherited retinal disease, affecting 1 in 3000-7000 individuals. Mutations in over 60 known genes have been found to be causative factors of RP, among which mutations in the rhodopsin (*RHO*) gene are the most common cause and are associated with 25%~35% of autosomal dominantly inherited RP (adRP), affecting 7,500 patients in US and 12,100 patients in EU and UK. More than 150 different mutations in the *RHO* gene have been identified to cause RHO-adRP with progressive loss of rods followed by loss of cones and the apoptosis of the photoreceptor cell, resulting in the loss of the visual function. Currently, there are no curative therapy for RHO-adRP. Here we developed a gene editing based therapy strategy to knockout the endogenous *RHO* gene independent of the mutations via proprietary compact CRISPR/Cas12 system then introduce a normal exogenous copy to maintain the rod normal function via single AAVs - a "knockout and replace" therapy to treat RHO-adRP. **Methods**: We constructed a humanized RHO mouse model, with part of the mouse *Rho* gene

sequence replaced with human *RHO* gene sequence. An all-in-one AAV vector, comprising a rod-specific promoter fragment, a miniature CRISPR-Cas12 system and an optimized exogenous human *RHO* cDNA fragment (exo-RHO) with the highest expression efficiency (Cas12-hRHO), was constructed to develop a therapeutic strategy that rescues the visual function by knocking out the endogenous *RHO* gene (endo-RHO) independent of the mutations and supplementing with an exogenous human *RHO* copy. 6-week-old humanized RHO mice were subretinally injected with AAVs. We further tested this approach in human retinal explants and the cynomolgus monkey retina. **Results:** In the humanized RHO mice, Cas12-hRHO efficiently edited the endo-RHO, resulting in ~70% mRNA knockdown. Furthermore, the exo-RHO mRNA was successfully expressed to ~50% of the normal level (Figure 1A). Similar results were also observed in human retinal explants infected with Cas12-hRHO. Moreover, efficiency of Cas12-hRHO was tested in the cynomolgus monkey retina by subretinal injection (Figure 1B). Although our gRNAs had mismatches with the cynomolgus monkey genome, we still observed approximately 20% editing efficiency. The amount of endogenous *RHO* transcripts decreased by nearly 70%, and the amount of exogenous *RHO* transcripts was approximately 150% that of the endogenous controls. **Conclusions**: Our study provides an alternative CRISPR/Cas12-based treatment for RHO-adRP. Further demonstration of *in vivo* treatment effects using the disease mouse model will verify the potential of Cas12-hRHO as a therapeutic strategy for RHO-adRP with the potential to treat any of the over 150 dominant gain-of-function mutations with a single injection.

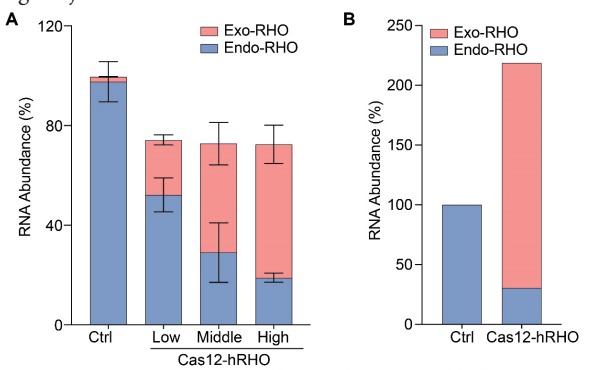

Figure 1. Expression of endo/exo-RHO mRNA in mice and NHP. **A**, Expression of endo/exo-RHO mRNA in humanized RHO mice subretinally injected with Cas12-hRHO with different doses. Ctrl, humanized RHO mice injected with vehicle. n= 4-6/group. Data was shown as mean ± s.e.m. **B**, Expression of endo/exo-RHO mRNA in NHP subretinally injected with Cas12-hRHO. Ctrl, NHP eye injected with vehicle. n= 1/group.

713 Restoration of Retinal Function and Structure in an *Rpe65*-Deficient Murine Model via Gene Replacement Therapy

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Background: Mutations in *RPE65* block the visual cycle, resulting in a congenitally inadequate supply of chromophore to both rod- and cone-photoreceptors. The *RPE65*-mediated inherited retinal dystrophy (IRD) spectrum therefore exhibits common clinical findings, initially characterized by night blindness that begins in early childhood followed by progressive degeneration of retinal structure and function. Here, we reported HG004, a novel investigational AAV based-gene therapy drug, for the treatment of *RPE65*-IRD and evaluated its efficacy in a *Rpe65*-deficient murine model. **Methods:** We independently-developed a *Rpe65*-deficient murine model by proprietary CRISPR genome-editing system. These animals (1 to 2-month-old or 10-month-old) were randomized into groups and administrated with either HG004 or vehicle by a single subretinal injection. Efficacy was evaluated by retinal optical coherence tomography (OCT), electroretinography (ERG), and histochemical staining. **Results:** Scotopic b-wave responses in all treated groups were significantly recovered at all time points, compared with vehicle-injected control eyes of *Rpe65*$^{-/-}$ mice ($p<0.01$) after the injection of HG004 (Fig. 1a). The restoration ratio at 3×10^8 vg/eye was over 60% of the wildtype (WT) level, and the durability of the therapeutic effect maintains for at least 34 weeks (Fig. 1a). At week 35 post-injection, the retinal thickness of *Rpe65*$^{-/-}$ mice treated with HG004 at 3×10^8 vg/eye could be maintained at about 85% of the WT level (Fig. 1b), compared with the untreated eyes (67% of the WT level). Furthermore, at week 15 post-injection of HG004 at 6×10^8 vg/eye, the ERG amplitude of the treated eyes in 10-month-old *Rpe65*$^{-/-}$ mice could recover to 38.7% of WT mice at the same age (Fig. 1c). The retinal thickness of these eyes could be maintained at about 62% of the WT level, while the untreated eyes were only 53%. In contrary to the abnormalities in the retinal structure of untreated *Rpe65* deficient mice, the photoreceptor cells of the treated eyes were neatly arranged, and the OS density was consistent with that of the WT animals at week 16. **Conclusions:** Our findings demonstrated that HG004 could significantly restore the function of RPE cells and photoreceptor cells and maintain the integrity of retinal structure in *Rpe65*$^{-/-}$ mice. Furthermore, the durability of the therapeutic effect was sustained for at least 34 weeks. Although the retina of 10-month-old *Rpe65*$^{-/-}$ mice had shown irreversible photoreceptor cell degeneration, the treatment of HG004 could still restore the functions of the remaining photoreceptor cells and RPE cells, resulting in the prevention of the retinal outer segment disc disorder and further degeneration of the photoreceptor cells. These results strongly support the hypothesis that HG004 may provide benefits to *RPE65*-IRD patients. Importantly, our data generated by *Rpe65*-deficient murine model support the recent HG004 IND clearance by US FDA.

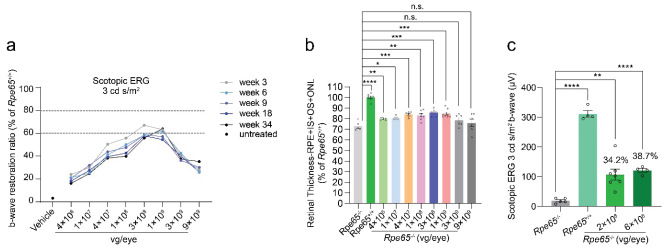

Figure 1. Efficacy of HG004 in *Rep65*-deficient murine model

714 COMET-Mediated Delivery of C^{3}DNA in Human Cell-Based *In Vitro* Models of Retinal Pigment Epithelium

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Introduction Inherited retinal diseases, such as Stargardt disease (STDG1), are a large group of genetically and clinically heterogeneous conditions which lead to blindness. Stargardt disease (*ABCA4*- related) is the most common form of juvenile macular dystrophy characterized by the accumulation of lipid-rich deposits (also known as lipofuscin) in the retinal pigment epithelium (RPE), RPE atrophy, and photoreceptor cell death. It was shown recently that lack of *ABCA4* function disrupts lipid homeostasis in the RPE cells leading to lipofuscin formation, which is a hallmark of STDG1. STDG1 is autosomal recessive and polymorphic with > 900 known mutations in the human *ABCA4* gene. Delivery of full-length wild type copy of human *ABCA4* gene may provide an ability to treat the spectrum of *ABCA4*-related retinopathies in a mutation agnostic manner. However, the large size of the wild type human *ABCA4* gene makes its delivery unamenable to AAV mediated delivery. Intergalactic's proprietary non-viral C^{3}DNA (covalently closed circular DNA) allows for the design and delivery of DNA payload beyond the size limitation of canonical viral cargo. Additionally, Intergalactic's proprietary electro-transfer system, COMET, enables the local delivery of genes to the relevant cell type. To assess the functional effect of delivery of full length *ABCA4* to the retinal cells in vitro, it is important to establish a relevant cellular model system. The *Abca4* knock-out mouse model shows lipofuscin accumulation in the RPE. To recapitulate the human RPE dysfunction phenotype, we generated a CRISPR knockout of the *ABCA4* gene in human inducible pluripotent stem cells (IPSCs) and differentiated them to RPE cells (iRPE). Our goal was to utilize the iRPE cells that were null for *ABCA4* to investigate the effect of COMET-mediated delivery of C^{3}DNA expressing full length, wildtype ABCA4 protein on RPE function. **Methods** Wild type (WT) iRPE cells were derived from a commercially available IPSC cell line using previously reported methods. CRISPR gene editing was used to generate an *ABCA4* knock-out (*ABCA4* KO) iRPE cell line that was null for *ABCA4*. Both WT and *ABCA4* KO cell lines were confirmed to be differentiated RPE cells using flow cytometric analyses for progenitor markers, PAX6 and MiTF, and mature RPE markers, BEST1 and RPE65. Additionally, the two cell lines were assessed for RPE function based on their ability to phagocytose labeled photoreceptor outer segments. WT and ABCA4 knock out iRPE cell lines were transfected with C3DNA expressing full length human *ABCA4* gene using COMET. Post-transfection, both WT and ABCA KO iRPE cultures were assessed for transepithelial electrical resistance (TEER), phagocytosis, protein expression of ABCA4, and lipid dysregulation. **Results and Discussion** There was no difference between wild type and *ABCA4* KO cells in barrier function with TEER and phagocytic uptake of FITC-labeled photoreceptor outer segments. Assessment of lipid dysregulation in *ABCA4* KO cells showed an increased intracellular accumulation of cholesterol as indicated by filipin staining and a significant increase in neutral lipid formation, indicated by BODIPY staining. These data suggest that *ABCA4* may have a role in lipofuscin formation, which is rich in lipids. Based on the above results, *ABCA4* KO RPE cell line could serve as an appropriate in vitro model system to assess the effect of COMET-mediated delivery of C^{3}DNA expressing full length, wildtype *ABCA4* protein on RPE function.

715 *In Vivo* Production of Full-Length ABCA4 Protein Following Cre-Mediated Recombination from Dual AAV Vectors in ABCA4$^{-/-}$ Mice

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Autosomal recessive Stargardt disease is caused by mutations in the retina-specific ABCA4 gene. Decreased central vision due to loss of photoreceptors in the macula and increased fundus autofluorescence are hallmarks of the disease. Due to the limited packaging capacity of AAV vectors, the 6.8 kilobase human ABCA4 open reading frame cannot be packaged into a single viral particle. As an alternative, we developed a dual vector strategy that uses a Cre-mediated recombination between two partial sequences to produce full-length hABCA4. We were able to express full-length hABCA4 protein both in vitro via viral transduction of HEK293 cells and in vivo in photoreceptor cells after subretinal dual AAV injection into ABCA4$^{-/-}$ mouse eyes. We confirmed fidelity of Cre-mediated recombination of ABCA4 by sequencing the transgenic RNA from transfected HEK293 cells as well as from AAV-dosed animals. Immunohistochemistry results confirmed correct localization of recombinant hABCA4 protein into photoreceptor outer segments of dosed animals. Our results showed that Cre-mediated recombination of dual AAV vectors was safe and effective in delivery and expression of full-length ABCA4 in a knockout mouse model, paving the way for a novel therapeutic approach for treating Stargardt disease that could be used as a universal approach for multipartite large gene delivery.

716 ELOVL2 Gene Therapy: Photoreceptor Lipid Restoring Strategy to Treat Dry Age-Related Macular Degeneration (AMD)

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Dry age-related macular degeneration (dry AMD) is a slowly progressing debilitating retinal disease which leads to the loss of macular cone photoreceptors. It is the third leading cause of blindness in the world with a prevalence similar to that of cancer and more than twice that of Alzheimer's. People with advanced stages of dry AMD struggle with everyday tasks such as reading and facial recognition. Currently there are no approved treatments. Elongation of very-long-chain fatty acids-like 2 (ELOVL2) enzyme is the rate limiting elongase involved in the elongation of long-chain (C22->C24) polyunsaturated fatty acids (LC-PUFAs). ELOVL2 is involved in the production of docosahexaenoic acid (DHA) 22:6n-3 as well as very-long-chain PUFAs (VLC-PUFAs). DHA is the most abundant polyunsaturated fatty acid

(FA) in mammals, constitutes approximately 35% of total FAs in the retina and is the most abundant FA in rod outer segments (50-70% of FAs). Within photoreceptor outer segment disk membranes, these LC-PUFAs provide a crucial and specialized environment for the proper functioning of opsins in the visual pathway. Knockout of ELOVL2 in mice, results in a phenotype similar to AMD; diminished ERG, drusen formation and complement accumulation within the retina of young mice. As mice age, the ELOVL2 promoter becomes increasingly methylated resulting in decreased ELOVL2 expression. In humans, a CpG island within the promoter of ELOVL2 becomes progressively methylated with age and is one of the most robust biomarkers of age. Diminishing ELOVL2 expression and the concomitant decline in DHA and VLC-PUFA production associated with aging, we believe is a major factor underlying susceptibility to AMD. To test this, we designed and constructed a series of optimized ELOVL2 expression cassettes as candidate therapeutics for dry AMD related vision loss and we pursued both viral and non-viral approaches. One-year old C57BL/6J mice were treated with a single subretinal AAV8-optELOVL2 administration or with saline vehicle in the contralateral eye and followed for six months. Compared to vehicle, treated eyes were protected against aging related loss of vision as evidenced by: 1) ERG a and b waves of greater magnitude and shorter latency and 2) reduced thinning of the outer retina photoreceptor layer (a hallmark of dry-AMD) as measured by OCT. Efficacy at higher dosages was limited by toxicity which we believe to be promoter related. Second-generation viral constructs which are high expressing and better tolerated were also tested and provided protection from thinning of the outer retina photoreceptor layer in mice. The results from a non-human primate study using AAV-ELOVL2 are currently being collected and analyzed and will be presented. To overcome some of the limitations of AAV as a delivery vehicle, a novel method for creating DNA constructs with payloads of any size is being developed. These synthetic constructs have been delivered non-virally using lipid nanoparticles and have demonstrated strong and durable expression *in vitro*. The results from a planned mouse study will be presented to further evaluate the efficacy of this new method.

717 Best Time for Adeno-Associated Virus (AAV) Neutralizing Antibody (NAb) Prescreening of NHPs Prior to Gene Therapy Studies

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Following recent approval of AAV based drugs by FDA, a huge increase in the number of gene therapy trials employing AAV based viral vectors has been observed. Efficacy of AAV drugs can be compromised by the presence of neutralizing antibodies (NAb) in both humans and NHPs as a result of immune responses to previous exposures to AAV. Therefore, it's very important to prescreen NHPs prior to selection for gene therapy studies as NAbs may interfere with the biodistribution of the AAV drugs, potentially impacting the outcome of clinical trials. Prescreening of NHPs is routinely performed at the vendor site or during quarantine at the study site; however, determining how far in advance to test these NHPs to avoid seroconversion between testing and the start of a study remains an important question. To begin to answer this question, we performed two separate studies tracking seroconversion in NHPs. In the first study, 100 cynomolgus macaques were sourced from a single Asian supplier. They were housed in separate rooms and occasionally were grouped with cohorts from other suppliers. Sera was collected from NHPs at 0, 4 and 7 months post arrival. The sera samples from 20 NHPs, 12 males and 8 females, were tested by AAV2, AAV8, AAV9 and AAVrh74 NAb screening assays at sample dilutions of 1/10, 1/20 and 1/40. While the majority of NHPs showed no significant change in AAV NAb titer over the course of the study, a small number of animals showed an increase in NAb titer greater than 4-fold in 4-7 months. In a second study, 300 male and 300 female cynos from a single supplier were screened on arrival (week 0) with 76 of them negative for AAV8 NAbs. Another round of AAV8 NAb screening was performed at week 8 with 13/76 sera showing seroconversion. Additionally, these 13 seroconverted NHPs (week 0 vs 8) were screened by AAV2 and AAV9 NAb assays showing a reduced rate of seroconversion for these two serotypes indicating seroconversion for different serotypes was not similar in individual NHPs. Both studies confirmed seroconversion occurs in NHPs with time. This suggests that, in addition to prescreening, a second round of confirmatory screening is highly recommended, preferably within a month prior to beginning gene therapy studies.

718 Neutralizing Antibody Seroprevalence of Six Adeno-Associated Virus Serotypes Relevant to Gene Therapy in the United States

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Introduction: Adeno-associated virus (AAV) mediated gene therapy for several indications have been approved, although pre-existing neutralizing antibodies (NAbs) against AAV serotypes can interfere with efficacy. This subset analysis of an international, noninterventional, observational, retrospective, cross sectional study focused on the NAb prevalence of 6 AAV serotypes in individuals aged ≥16 and <16 from the United States. Methods: Serum samples from the Pfizer Biobank were obtained from participants that were enrolled in previous non-gene therapy clinical studies conducted between 2015 and 2019. Matching participant-level data on demographics and clinical characteristics were obtained from each clinical trial database. NAb seroprevalence for 6 AAV serotypes (AAV1, AAV5, AAV6, AAV8, AAV9, AAVRh74var[AAV-Spark100]) was estimated at a range of dilutions (from 1:1 to 1:50,000), and NAb titer levels were determined using anti-AAV NAb assays based on cell transduction inhibition (ID50). Results: There were 50 samples from patients ≥16 years of age. At 1:1 dilution, NAb prevalence was lowest for AAV9 (48%) and AAVRh74var [Spark 100] (48%); 86% for AAV1, 68% for AAV5, 66%

for AAV6, 50% for AAV8. At 1:2 serum dilution, NAbs against AAV1 and AAV6 were the most prevalent (48% and 54%, respectively). At 1:2 and subsequent dilutions, AAV5 had the lowest seroprevalence among the AAV serotypes. At 1:4 serum dilution, NAbs against AAV5 (18%) and AAVRh74var (34%) presented the least prevalence, while NAbs against AAV1 remained the most prevalent. At 1:2,000, 1:10,000, and 1:50,000 dilutions all NAbs against the 6 AAVs were undetectable. There were 31 samples from patients <16 years of age. At 1:1 dilution, NAb prevalence was lowest for AAV8 (39%) and AAV9 (39%); 68% for AAV1, 55% for AAV5, 52% for AAV6, 42% for AAVRh74var. At 1:2 serum dilution, NAbs against AAV1 (42%) was the most prevalent and least prevalent for AAV5 (26%). Similar to adults, at 1:2,000, 1:10,000, and 1:50,000 dilutions all NAbs against the 6 AAVs were undetectable. Conclusions: The seroprevalence of NAbs against 6 different AAV serotypes in the US was determined using a robust cell transduction inhibition assay. Overall, children exhibited lower seroprevalence compared with adult samples, consistent with natural environmental exposure. Our results indicate variation within the US across different AAVs, with implications for clinical gene therapies.

719 Optimized CRISPR Engineering of the IgH Locus in Non-Human Primate Hematopoietic Cells for Induced Humoral Immunity

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B cell editing has enabled expression of engineered antibodies conferring anti-viral protection in murine models. CRISPR/Cas systems can target the immunoglobulin heavy chain (*IgH*) locus, replacing endogenous antibodies with transgenes delivered by viral vectors[1]. While this approach could replace repeated antibody infusions, it remains unclear how long a gene edited B cell immune response can last. A potential solution is engineering hematopoietic stem and progenitor cells (HSPC), precursors to all B cells. A large-animal model such as non-human primates (NHP) could permit more clinically relevant, autologous studies with infectious challenge. Here, we evaluated editing to introduce transgenic sequences at the *IgH* locus of NHP. We isolated HSPC and sequenced the *IgH* locus from two different species, *Macaca mulatta* and *Macaca nemestrina*, to design optimal CRISPR components. We observed 99.2% identity between the two species and 84.2% identity to human sequences in the *IgH* constant region. To identify optimal CRISPR guide RNAs, sequences predicted to result in ≥3 bp deletions were selected as preferential for homology-directed repair (HDR) outcomes[2]. A total of 10 RNA guides out of hundreds were synthesized for testing, n=5 each for Cas9 and Cas12a. We purified CD34$^+$ HSPC using immunomagnetic separation on whole bone marrow aspirate from healthy donor animals (n=2). HSPC were electroporated using equal ribonucleoprotein concentrations for each nuclease. Next-generation sequencing demonstrated higher indel frequencies with Cas12a than Cas9, with editing rates up to 2.3% when accounting for background mutations and a targeted quantification window (Fig.a). Indel types observed for Cas12a aligned with higher likelihood of HDR events. To test this, a DNA template (JMPC01) encoding a GFP reporter was used to evaluate HDR at the optimal Cas12a target site. This template was co-delivered with Cas12a via electroporation in an NHP B cell line (LCL 8664) as plasmid DNA, double-stranded DNA (dsDNA), and end-biotinylated dsDNA (Bio5P dsDNA), resulting in 1.0%, 2.6%, and 1.9% GFP+ cells at day +10, respectively (Fig.b). We are now applying this editing strategy in primary NHP HSPC to assess engraftment and differentiation into B cells in the MISTRG mouse strain. Based on experience in primatized MISTRG mice, B cell progeny will be evaluable in the next month. Further studies in pre-clinical NHP models will assess the safety and efficiency of this approach for achieving long-term humoral immunity in diseases for which there are no vaccines.

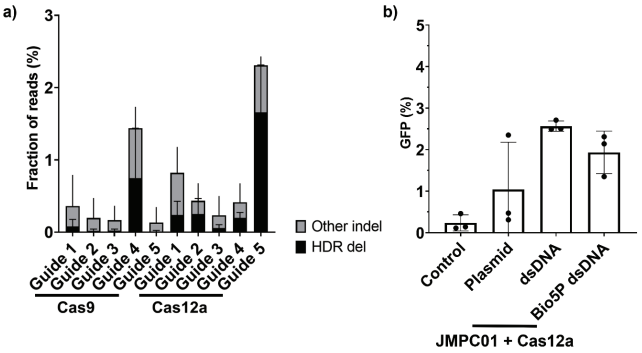

Figure. a) Frequency of indels and HDR-like deletions (HDR del ≥3 bp deletions) at the cut site in HSPC. b) GFP expression in LCL 8664 at day +10, comparing JMPC01 with Cas12a to control without nuclease.
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720 Genetic Vaccine Induced T Cells Can Partially Control SARS-CoV-2 Replication

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Vaccine or infection induced SARS-CoV-2-specific neutralizing antibodies (NAbs) show a poor cross reactivity between SARS-CoV species and SARS-CoV-2 variants and often fail to protect against infection. However, protection against severe disease and death suggests a protective role for cross-reactive T cells. To better understand the role of Nabs and T cells, we generated genetic and protein vaccines containing the complete spike, the receptor binding domain (RBD), the membrane (M) and/or nucleoprotein (N) that induced only T cells, or both NAbs and T cells. In three challenge models with homologous or heterologous challenge, high levels of vaccine induced SARS-CoV-2 NAbs did not protect against infection or histological signs of disease but conferred a rapid viral control and a mild limited histological damage. In the absence or low levels of cross-reactive NAbs, vaccine-

primed T cells were able to partially control viral replication to promote a NAb recall-responses. T cells alone did not protect against histological damage, most likely due to more extensive viral spread the absence of NAbs, and instead a partial control mediated by the elimination of infected cells by T cells. Overall, vaccine or infection induced Nabs will most likely never confer a long-term sterile immunity against SARS-CoV-2, as has become evident from real life studies. More realistically, multi component SARS-CoV vaccines should strive for both broadly cross-reactive NAbs and T cells. Importantly, cross-reactive T cells primed by multi component vaccines and/or natural infection, may offer a long term protection against severe disease and death during the current and future pandemics.

721 Extreme Inter-Individual Variability of Subretinal AAV-Induced Immune Response Even in a Highly Standardized Context

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Introduction. Ocular gene therapies that use adeno-associated viruses (AAVs) to deliver genes have been successfully applied in some inherited retinal diseases. However, inflammation resulting from immune responses to the AAV or the transgene remains an important concern. Anti-transgene and anti-capsid immune responses has been reported in some patients who received AAV gene therapy, which adversely can affect safety and efficiency of the therapy. At present, immunomonitoring and finding pertinent biomarkers are crucial challenges for patient management. Heterogeneity in the strength of the immune response between patients is traditionally thought to be the consequence of genetic background diversity and previous exposure to different pathogens. In a syngeneic murine model, we have previously reported that a single subretinal injection of AAV8 induces a systematic anti-transgene T-cell response, but with with different levels of intensity. Here, we analysed complementary innate and adaptive immune components to have a better understanding of the immune consequences subsequent to a subretinal AAV injection and the degree of individual diversity in a standardized context. Methods. Transgene cassette encoding the HY male antigen, containing MHC class I- and MHC class IIrestricted T-cell epitopes, immunodominant in female H-2b background, was packaged into AAV8 under the ubiquitous PGK promoter. Several experiments were done with a single subretinal injection of high dose AAV8-HY was performed at day 0 in immunocompetent adult female wild type C57BL/6 (H-2b) mice. At day 21, RT-droplet digital PCR (ddPCR), ELISpot, in vivo cytotoxicity and ELISA were done to evaluate the transgene expression, antigen presenting cell markers, T-cell responses and antibody production. Principle component analysis, correlations, and diversity index have been calculated based on the data from each readout. Results. Surprisingly, we found a very strong variability of the immune response between mice of the experiments, despite a steady local and systemic immune reactivity following the subretinal injection of AAV8-HY. Indeed, none of the mice display the same immune response profile albeit the identical genetic background, injected product, housing conditions, and surgery being performed by the same individual. Conclusions. We provide experimental evidence on the extreme inter-individual variability of local and systemic immune responses following a subretinal AAV injection on syngeneic mice and in a highly standardized context. This suggests that in patients subjected to ocular gene therapy, in addition to variable genetic backgrounds and different immune history, immunomonitoring should take into account that inter-individual immune responses are basically very heterogeneous, and no individual immunological parameter can predict the evolution of the immune response behaviour alone.

722 Enabling *In Vivo* Therapeutic Antibody Production through Hematopoietic B Cell Engineering

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Persistent therapeutic protein production has tremendous potential for a myriad of malignant, genetic and infectious diseases. B cells are among the most proficient protein-secreting cell types in the body and are highly specialized for expression of antibodies. Recent studies demonstrate that *ex vivo* and *in vivo* gene-edited human and murine B cells can efficiently produce therapeutic proteins, including antibodies. However, few studies have explored this approach in immunocompetent large animal models, which most closely model and predict therapeutic potential in patients. Therefore, we have been developing a nonhuman primate (NHP) model of B cell gene therapy to assess the function and persistence of engineered B cells *in vivo*. Considering our longstanding experience with persistent HIV-1 acquisition in the NHP model, our experiments have focused on the reprogramming of distinct hematopoietic cell types with HIV-specific broadly neutralizing antibodies (bNAbs). NHP hematopoietic stem and progenitor cells (HSPCs) and B cells were modified *ex vivo* with bNAb-encoding lentiviral vectors (LV) or CRISPR-Cas9/AAV6 donors, respectively. Both LV and AAV6 donors encoded VRC01, an anti-HIV bNAb that has been extensively investigated in clinical studies. AAV6-VRC01 donors were targeted to the endogenous immunoglobulin heavy chain (IGH) locus. PCR- and flow cytometry-based methods were used to quantify gene-modified cell products *ex vivo* and their persistence *in vivo*, while antibody production was assessed by ELISA. A pilot experiment focused on autologous transplantation of VRC01 bNAb LV-modified HSPCs. Although modified cells and VRC01 mRNA expression persisted, secreted VRC01 antibody was detected at low concentrations in the blood before declining to undetectable levels 2 months post-transplant. Anti-drug antibodies (ADA) directed against the exogenously expressed proteins were also detected. Considering these results, we then investigated B cell editing as an alternative approach to augment bNAb expression and persistence while decreasing immunogenicity issues in the NHP model. The editing approach demonstrated an MMEJ:NHEJ ratio associated with efficient HDR and resulted in 80-90% indels efficiency. Integration of the VRC01 donor at the targeted IGH locus was confirmed by in-out PCR. Up to 15% of the B cells expressed VRC01 bNAb at the

cell surface as detected by flow cytometry 5 to 8 days post-editing. Our data demonstrate efficient macaque B cell engineering for anti-HIV bNAb production and lay the foundation for the evaluation of this approach in our immunocompetent NHP model. Targeted modification of the IGH locus in B cells provides many advantages over lentivirus modifications or repeated antibody injections such as acquisition of tolerance, non-immunogenic glycosylation pattern, antigen-inducible protein production, affinity maturation and antibody delivery to tissues of interest. The recent development of *in vivo* editing approaches designed to enhance scalability and feasibility in low and middle income countries could greatly accelerate the development of B cell-based therapies. Although these studies focused on treatments for people living with HIV, our approach holds promise for many chronic diseases in which standard of care includes repeated injections of antibody protein.

723 Neonatal Fc Receptor Inhibition Enables Adeno-Associated Virus Vector Gene Therapy Despite Pre-Existing Humoral Immunity

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Advances in adeno-associated virus (AAV)-based gene therapy are transforming our ability to treat rare genetic disorders and address other unmet medical needs. However, the natural prevalence of anti-AAV neutralizing antibodies (NAbs) in humans currently limits the population who can benefit from AAV-based gene therapies. Neonatal Fc receptor (FcRn) plays an essential role in the long half-life of IgG, a key NAb. Researchers have developed several FcRn-inhibiting drugs, including monoclonal antibodies, to treat autoimmune diseases, as inhibiting the FcRn-IgG interaction can reduce circulating IgG levels to 20%-30% of the baseline. We evaluated the ability of one such monoclonal antibody, M281 (nipocalimab), to reduce pre-existing NAb levels and thereupon enhance gene delivery to the liver and heart upon systemic administration of AAV gene therapy in mice and rhesus macaques (newborns and adults). M281 treatment prevented human NAb-mediated reduction in liver transduction by 83% (p=0.0039) following intravenous AAV administration in humanized FcRn transgenic mice to which pooled human IgG was passively transferred. In newborn rhesus macaques, M281 rapidly eliminated maternally derived anti-AAV antibodies and NAbs to below the detection limit. In adult rhesus macaques, M281 transiently reduced pre-existing NAb titers from 1:40-1:80 to 1:10 or lower along with total IgG levels; M281 treatment also enhanced gene delivery to the liver by 7.4-fold and to the heart by 61.4-fold after intravenous AAV administration in NAb-positive animals. These results indicate that mitigating pre-existing humoral immunity via disruption of the FcRn-IgG interaction may render AAV-based gene therapies effective in NAb-positive patients.

724 Preliminary Study of AntiAAVrh74 Seroprevalence Following Gene Transfer

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The increasing number of gene therapy studies and exclusion of candidates from clinical trials highlights the potential importance of pre-existing antibodies targeting viral capsid. Viral antibodies block transduction and preclude expression of the transferred gene. Provoked immune response could also be a threat to safety. Testing patients for pre-existing antibodies, either neutralizing (Nabs) or total/binding (TAbs/BAbs), prior to gene transfer is a critical step in the initiation of a clinical trial. Previous studies assessed the seroprevalence of antibodies against different AAV subtypes in the general population and in patients receiving gene transfer. Our gene therapy studies for muscular dystrophy (DMD, LGMD) have currently shown promise for efficacy and safety, but we don't know if vector shedding post gene delivery affects the antibodies titer in family members of trial participants. This is particularly relevant for siblings who might become next gene therapy candidates. This pilot study included 115 subjects divided in three separate cohorts. Group 1 consisted of hospital personnel without known prior work exposure to rAAVrh74 (control group, 23 subjects). Group 2 were hospital-based personnel with known work exposure to rAAVrh74 (through pre-clinical and clinical studies, or direct contact with patient's specimen, 35 subjects) and Group 3 were relatives of patients receiving rAAVrh74 as part of a gene transfer trial (57 subjects). Enzyme-linked immunosorbent assay (ELISA)-based detection was used to identify anti-AAVrh74 total antibodies. Titers of 1:50 and 1:400 were reported, considering the range of titers used for enrollment in different trials. We found that parents of patients receiving gene transfer had a 3-fold greater risk of AAVrh74 seropositivity at 1:50 compared to the control group: 33.3% AAVrh74 positive (19/57 subjects) vs 13% negative (3/23 subjects), with a relative risk of 2.6%. Additionally, we observed that there were no statistically significant differences between two hospital personnel cohorts, non-exposed (Group 1) and exposed (Group 2), in terms of antibody positivity to AAVrh74. Finally, antibody titers at 1:400 were not different between Groups 1,2, or 3. These preliminary results serve as a caution to family members caring for children undergoing AAV gene transfer, warning that viral shedding following gene transfer can have an effect on the antibody titers. Prospective studies are on the way, with greater sample size, inclusive of pre- and post-treatment testing, and with the objective of establishing both the antibodies cut-off titers and the risk for acquiring immunity that could preclude gene transfer.

725 Specific Pharmacological Blockade of IRAK4 Suppresses AAV Induced Immunogenicity

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Immune responses to adeno-associated viruses (AAVs) pose a major challenge for successful clinical translation of gene therapy. Studies

based on several mouse models show that the innate immune DNA sensor TLR9 recognizes CpG-rich hypomethylated DNA in the transgene of AAV vectors. TLR9-triggered signaling cascade activates adaptive immune responses that ultimately lead to clearance of transgene induced cells by T cell mediated cell cytotoxicity. In line with this, data from hemophilia clinical trials reveal that reduction in total number of CpG bases reduces the need for pharmacological immunosuppressants and show reduced cytotoxic T lymphocyte responses in hemophilia patients. While broadly acting immune suppressants have been successful in improving AAV delivery in clinical trials, they still result in loss of transgene expression and present with side effects and risk of opportunistic infections. Developing vectors with no CpG nucleotides is challenging because non-codon optimized vectors (i.e., vectors with no or low CpG content) typically exhibit poor transgene expression. Hence, what is needed is a different class of immune modulators that show enhanced specificity and low side effects. IRAK4 is a key kinase within the TLR9 pathway, which is critical for mounting an effective innate immune response. We hypothesized that inhibiting IRAK4 by kinase inhibitors or degraders should result in a specific blockade of TLR pathway resulting in immune modulation favorable for AAV treatment. To this end, we tested commercially available inhibitors/degraders of IRAK4 to block TLR9 signaling in-vitro. We utilized primary human monocyte derived dendritic cells (moDCs) that were either untreated or pre-treated with IRAK4 inhibitor/degrader before AAV infection. We have previously described the detection of a consistent statistically significant increase in cytokine 'signature' (IL1b, TNFa and IL6) resulting from AAV administration to moDCs. Here, we observed that moDCs pre-treated with IRAK4 inhibitor or degrader showed significant reduction in AAV induced signature cytokines as compared to cells that were not treated with IRAK modulators. In addition, both the IRAK4 inhibitor and degrader were not cytotoxic to primary human dendritic cells and resulted in more than 50% IRAK4 degradation. To further extend these novel findings we are testing different doses of both IRAK4 inhibitor/degrader delivered one time either as pre-treatment (prior to AAV infusion) in an in-vivo mouse model of AAV immunogenicity. Applicability of this strategy has the promise to be pan-indication, such as Huntington disease, metachromatic leukodystrophy, amyotrophic lateral sclerosis, hemophilia A, and enable successful clinical translation of AAV gene therapy.

726 Neutralizing Antibody Depletion with Imlifidase - A Potential Way to Enable AAV Based Gene Therapy in Seropositive Patients

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Pre-existing neutralizing antibodies towards adeno associated virus (AAV) based gene therapy are a major problem, as they are present in 30-60% of patients, making them ineligible for successful gene therapy treatment. However, these neutralizing antibodies can be effectively depleted by imlifidase, an IgG cleaving cysteine protease originating from *Streptococcus pyogenes*. Imlifidase, with the tradename Idefirix®, has been granted a conditional marketing authorization in Europe* for desensitization of highly sensitized adult kidney transplant patients. In addition, there are several ongoing clinical trials in a variety of indications, with IgG antibodies and Fc-mediated effector responses as their common denominator. More than 99% of all IgG is efficiently cleaved within a few hours after dosing with one or two doses of the clinically used dose of 0.25 imlifidase mg/kg body weight. Newly synthesized IgG starts to show 5-7 days after dosing of imlifidase, thus creating an antibody-free window of approximately one week. IgG levels are normally restored to their initial levels after 2-6 months. Imlifidase has demonstrated an acceptable safety profile in clinical trials in kidney transplantation, anti-GBM disease and Guillain-Barré syndrome (GBS) with the most common adverse events being infection (16.5%) and infusion related reactions (5.6%), resolving within 90 min. The most common treatment related serious adverse events reported in this population were pneumonia (6%) and sepsis (4%). There were no serious adverse events reported in all 35 healthy subjects treated with imlifidase. All in all, antibody depletion with imlifidase could offer a simple and elegant way to enable gene therapy in seropositive patients with AAV antibodies and supports the rational for further clinical studies. The therapeutic effects of imlifidase established in various indications are thus being further evaluated in AAV based gene therapy in collaboration with gene therapy companies, including Sarepta Therapeutics and AskBio.
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727 Overcoming the Effect of Previous Enzyme Replacement Therapy on the Detection of Anti-Transgene Protein Antibodies after Treatment with Gene Therapy

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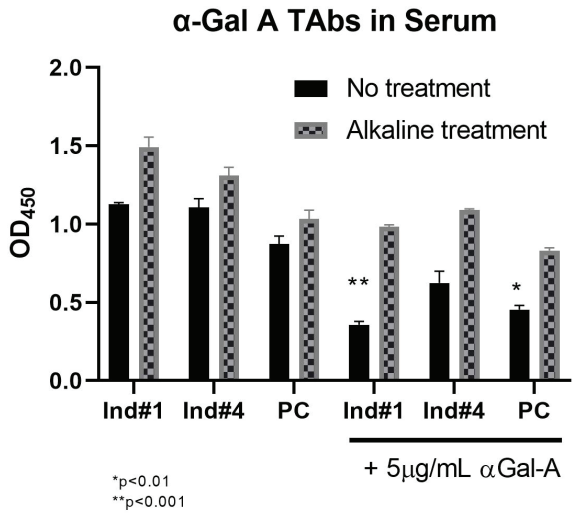
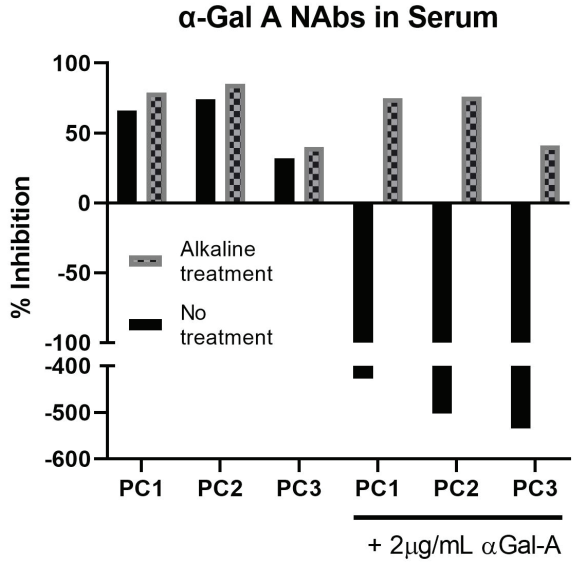
The increasing interest of *in vivo* gene therapy using recombinant adeno-associated virus (AAV) brings new bioanalytical challenges for assessing immunogenicity. Many AAV gene therapies in the clinic target lysosomal storage disorders with patient populations that are being treated with enzyme replacement therapy (ERT). Patients often develop high levels of persistent anti-drug antibodies (ADA) to the recombinant enzyme products that may have an impact on later gene therapy treatments. As such, there is a need to appropriately measure ADA and its potential impact on pharmacokinetics, pharmacodynamics, safety, and efficacy. Additionally, it is important during gene therapy drug development to be able to monitor relative ADA levels to assess the induction of tolerance or treatment-boosted ADA production for patients with pre-existing ADA. Typical methods for detecting ADA to lysosomal enzymes are generally intolerant to low levels of circulating enzyme. In order to appropriately determine ADA levels, it is required to assess at washout, which is not possible after administration with AAV gene therapy. In patients with Fabry disease, treatment with gene therapy results in continuous expression of lysosomal transgene enzyme, alpha galactosidase A (α-Gal A), which can interfere with the detection of ADA and assessment of changes in ADA levels. In order to address this interference, novel assay methods were developed to improve the enzyme tolerance of our anti-α-Gal A total antibody (TAb) and neutralizing antibody (NAb) assays. Specifically, inclusion of a sample

pre-treatment with alkaline buffer dissociated α-Gal A ADA immune complexes and selectively denatured circulating α-Gal A, thereby leaving ADA intact and free for detection in our TAb and NAb assays.





Improvement of anti-α-Gal A antibody assays with the incorporation of an alkaline pretreatment step.The left figure shows improvement in TAb detection Fabry patient serum as well as positive controls. The right figure shows improvement in NAb detection in three different positive control antibodies. *p<0.01 ; **p<0.001 ; PC-Positive Control ; Ind-Individual With this novel approach we demonstrate an improvement in circulating enzyme tolerance to microgram per milliliter levels in serum for both the TAb and NAb assays, with elimination of enzyme interference in the NAb to the highest α-Gal A level tested. The alkaline treatment was able to remove enzyme interference with positive control antibodies as well as with Fabry patient samples with pre-existing ADA to ERT, thus leading to a more accurate detection of ADAs regardless of the on-board α-Gal A enzyme levels. This method provides high enzyme level tolerance and allows for the detection of changes in ADA level due to treatment or immune tolerance. Due to the similar nature and functional pH of lysosomal enzymes, we believe this method is applicable for accurate antibody detection for other lysosomal storage diseases and may be invaluable in determining the impact of AAV gene therapy on pre-existing anti-ERT immunogenicity.

728 CRISPR-Cas9 In Utero Correction of Murine Tyrosinemia is Not Impaired by Pre-Existing Maternal Immunity to Cas9 Endonuclease

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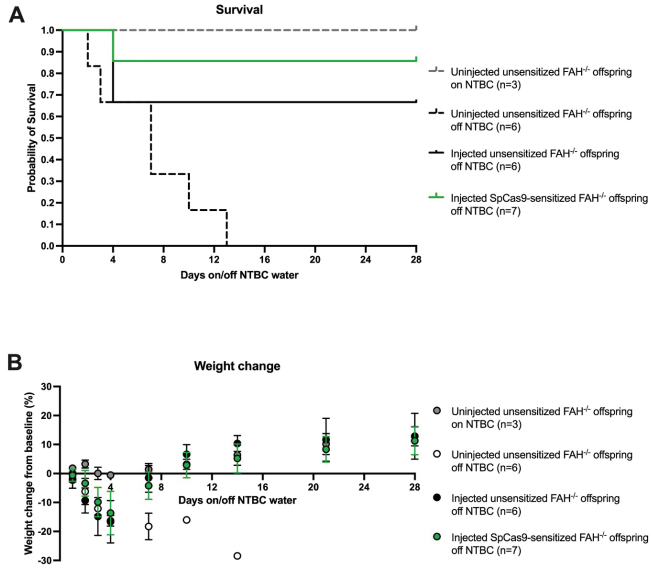
PURPOSE: In utero gene editing (IUGE) is an experimental treatment for inherited metabolic liver diseases including hereditary tyrosinemia type 1 (HT1). We have previously reported phenotypic correction in a mouse model of HT1 by means of in utero base editing of the *Hpd* gene, which codes the enzyme 4-hydroxyphenylpyruvate dioxygenase upstream in the tyrosine catabolism pathway, thereby preventing the lethal accumulation of toxic metabolites (Rossidis et al. *Nat Med* 2018). Pre-existing immunity to Cas9 endonuclease causes cytotoxic T cell-mediated destruction of CRISPR-Cas9-edited hepatocytes in adult mouse models (Li et al. *Mol Ther* 2020). Here we investigate whether pre-existing maternal immunity to Cas9 endonuclease impairs therapeutic gene editing of the fetal liver for HT1.
METHODS: Nitisinone (NTBC) is a medication that blocks the activity of 4-hydroxyphenylpyruvate dioxygenase. *Fah*$^{-/-}$ mice, a model of HT1, universally develop hepatitis, failure-to-thrive, and death in the absence of NTBC delivered via their mother's breast milk prior to weaning and in their water supply thereafter. We designed an adeno-associated virus serotype 9 (AAV9) containing the *Strep pyogenes* Cas9 endonuclease (SpCas9) transgene and a guide RNA for induction of indels in the murine *Hpd* gene (AAV9.SpCas9.Hpd). Maternal immunity to SpCas9 was induced by intramuscular injection of SpCas9 protein with saponin adjuvant 28 and 21 days prior to fetal injection of AAV9.SpCas9.Hpd, with successful induction of maternal cellular immunity confirmed by delayed-type hypersensitivity reaction. Gestational day 16 *Fah*$^{-/-}$ fetuses were injected with 10^{14} vg/kg of AAV9.SpCas9.Hpd. Offspring were maintained on NTBC until 6 weeks of age. Subsequent survival off NTBC was compared among experimental and control groups using the Log-rank (Mantel-Cox) test of equality of survival curves, and weight change from baseline was compared using analysis of variance. Fetal survival to birth was compared using Chi-square. Age-matched uninjected *Fah*$^{-/-}$ mice maintained on NTBC and taken off NTBC served as healthy and disease controls, respectively.
RESULTS: In utero injection of AAV9.SpCas9.Hpd resulted in 67% survival off NTBC among offspring of unsensitized *Fah*$^{-/-}$ dams (Figure 1A). Transient weight loss was observed in the first 4 days off NTBC with recovery to baseline by 7 days and continued weight gain above baseline thereafter (Figure 1B). Injected offspring of SpCas9-sensitized dams demonstrated equivalent survival off NTBC compared to injected offspring of unsensitized dams (86% vs. 67%, P=0.6). Weight change off NTBC followed a similar trajectory and was equivalent at all

timepoints (P≥0.32). Additionally, pre-existing maternal immunity to SpCas9 was not associated with adverse pregnancy outcomes and did not affect fetal survival to birth (53% vs. 67%, P=0.38). **CONCLUSION:** Pre-existing maternal immunity to SpCas9 does not impair in utero gene editing of the *Hpd* locus via AAV delivery of SpCas9 and *Hpd* targeting gRNA with rescue of the HT1 disease phenotype. This suggests a possible immunologic advantage for a fetal gene editing approach.



729 Non-Canonical Nucleic Acid Structures for Regulating the Immunostimulatory Effect of mRNA Therapeutics/Vaccines

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The modulation of PRRs-mediated recognition of exogenously delivered mRNA is important in the development of mRNA vaccine. The PRRs-mediated immune stimulation can be beneficial for the vaccine effect but can activate antiviral responses that limit antigen expression. In the context of RNA structures, long dsRNA and dsRNA with 5′triphosphate groups can be recognized by MDA5 and RIG-I, stimulating immune reaction but reducing antigen expression efficiency. To compromise this issue, current mRNA vaccines utilize the chemical modification technique for reducing immune stimulation and increasing antigen expression efficiency. To improve the current mRNA vaccine effect, it is crucial to find a way to induce immune stimulation without losing antigen expression efficiency. In this study, we developed non-canonical nucleic acid structures that can regulate the immunostimulatory effect of mRNA. The immunostimulatory effect of the developed nucleic acid structures was evaluated by measuring type I interferon (IFN) and secreted alkaline phosphate (SEAP) activities in the macrophage-like THP-1 Dual cells. By introducing the designed non-canonical nucleic acid structures into mRNA structure, the immunostimulatory effect of mRNA was controlled without chemical modification technique.

730 Liver-Specific Transgene Expression Attenuates Pre-Existing T Cell Responses but Fails to Prevent Transgene Loss

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Liver is viewed as a tolerogenic organ at steady-state, favoring induction of regulatory immune responses over pro-inflammatory responses. In the context of gene therapy, restricting transgene expression to the liver using liver-specific promoters has emerged as a potential strategy to attenuate *de novo* anti-transgene immune responses. However, while many studies have shown that liver-specific expression can promote immune tolerance to transgene in naïve mice, fewer studies have examined whether liver transgene expression can therapeutically suppress pre-existing anti-transgene immunity, and to what extent. Here, we investigated whether liver-restricted transgene expression could suppress strong pre-existing immunity in mice. Using an AAV8 vector, we expressed a foreign transgene (anti-human CD63 scFv fused to human acid alpha-glucosidase, "scFv:GAA") under a liver-specific transthyretin promoter, in either naïve Pompe (GAA-deficient) mice or mice previously immunized with scFv:GAA protein in Complete Freund's adjuvant (CFA). We found that while AAV-mediated liver expression protected previously naïve mice from developing antibody and T cell responses to scFv:GAA, even following CFA-adjuvanted immunization, antigen-experienced mice showed persistently high anti-GAA antibody titers, lymphocytic liver infiltrates (B and T cells), and partial transgene loss after AAV treatment. Functionally, high-titer pre-existing anti-GAA antibodies blocked therapeutic correction of tissue glycogen storage, including in heart and skeletal muscle. However, despite transgene loss and impaired glycogen clearance, there was evidence of a counter-regulatory response, including persistent PD-1 expression on liver T cells, upregulation of PD-L1 in the liver microenvironment, increased liver regulatory CD4+ T cells, and attenuation of systemic transgene-specific T cell responses by IFN-gamma ELISpot. Collectively, these data suggest while liver expression may suppress systemic transgene-specific T cell responses, it alone is insufficient to reverse entrenched, high-titer antibody responses and prevent liver transgene loss. While translatability to humans remains to be established, these results suggest the clinical benefit of liver-induced immune tolerance in liver-directed AAV gene therapies may depend on the strength of pre-existing immunity to transgene.

731 The HLA Class-II Immunopeptidomes of AAV Capsids Proteins

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Introduction: Gene therapies are using Adeno-associated viruses (AAVs) as vectors, but immune responses against the capsids pose challenges to their efficiency and safety. Helper T cell recognition of capsid-derived peptides bound to human leukocyte antigen (HLA) class II molecules is an essential step in the AAV-specific adaptive immunity.**Methods:** Using MHC-associated peptide proteomics, we

identified the HLA-DR and HLA-DQ immunopeptidomes of the capsid proteins of three different AAV serotypes (AAV2, AAV6, and AAV9) from a panel of healthy donors selected to represent a majority of allele usage.**Results:** The identified sequences span the capsids of all serotypes, with AAV2 having the highest peptide count. For all the serotypes, multiple promiscuous peptides were identified and displayed by both HLA-DR and -DQ. However, despite high sequence homology, there were few identical peptides among AAV2, AAV6, and AAV9 immunopeptidomes, and none were promiscuous.**Discussion:** Results from this work represent a comprehensive immunopeptidomics research of potential CD4+ T cell epitopes and provide the basis for immunosurveillance efforts for safer and more efficient AAV-based gene therapies.

732 Improving Lentiviral Gene Therapy by Inhibiting the Innate Immune Response

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With the advent of CAR-T therapy and other *in vivo* gene therapies like Zynteglo, lentiviral gene delivery has become a successful way to address severe chronic disorders. Lentivirus-derived vectors integrate their therapeutic transgenes into patient cells with generally high efficiency. However, sometimes extreme interpatient variability in lentiviral transduction efficiency is observed, and this can lead to suboptimal transduction in some cases (e.g. 34%-76% of patient cells transduced in clinical trial NCT03233854). While the adaptive immune response is known to hinder *in vivo* gene therapy, our research focuses on how the **innate** immune response of cells can negatively affect lentiviral transgene expression, even *ex vivo*. (Figure 1)



Figure 1. Potential inhibition of steps in the lentiviral gene delivery process by known AVGs. IFITM3 is a known inhibitor of the entry of lentiviral vectors into cells. AVGs IFI16 and Sp100 are involved in the silencing of foreign genetic material, even post-integration, and could potentially be responsible for the lack of transgene expression. MT1H is the only gene with any known antiviral role to significantly increase in expression because of lentiviral transduction. **In this study, we sought to elucidate the genes in the immune response that impact lentiviral gene delivery the most, then leverage this knowledge to design novel small molecule inhibitors for these genes. These inhibitors, once developed, will be inexpensive and easy to implement into lentiviral gene delivery processes for improved transduction efficiency.** Our study thus far has involved performing extensive mRNA sequencing to measure transcriptome-wide host cell gene expression of healthy donor PBMCs during the CAR-T manufacturing process and comparing this data to the transcriptome of HEK-293T cells, which have a consistently high transduction efficiency. The results indicated that several antiviral genes (AVGs) that are known to restrict lentiviruses were absent from HEK-293T cells but expressed at high levels in the PBMCs regardless of transduction throughout the entire *ex vivo* culture process. Analysis of the mRNA-seq data and extensive literature review yielded an ordered list of AVGs that are the most potent against lentiviral transgenes, and the top 4 were selected from this list for Cas9 KO and inhibition with siRNAs and/or small molecule inhibitors (SMIs), to determine if inhibiting these targets can improve transduction efficiency or transgene expression. (Table 1)

733 Xork-Fc, an Engineered IgG Protease, Shows Low Cross-Reactivity to Pre-Existing Antibodies in Human Serum and Enables Efficient AAV Transduction in an *In Vivo* Model of Passive Transfer of Neutralizing Human Serum

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Pre-existing neutralizing antibodies against AAV are highly prevalent and are a major exclusion factor for enrollment into many gene therapy trials. New strategies are needed to expand access of critical gene therapies to patients with pre-existing anti-AAV antibodies. Recently, bacterial-derived proteases specific for human IgG have been proposed as a method to transiently clear IgG from circulation and open a window within which AAV can be administered. We describe here the development of a novel IgG-specific protease, IdeXork (Xork). In contrast to IdeS, an IgG protease derived from the common human pathogen Streptococcus pyogenes, Xork is derived from a Streptoccocus species that is not known to infect humans. Consequently, levels of pre-existing antibodies against Xork are low or absent in normal human serum compared to the moderate-high levels of pre-existing antibodies that are prevalent against IdeS. We demonstrate that Xork cleaves human IgG in vitro with the same specificity and mechanism as IdeS. The in vivo activity of Xork was optimized by creation of an Fc fusion protein to extend its half-life. Near-complete (up to 97%) inhibition of AAV transduction in vivo by passive transfer of human serum with pre-existing anti-AAV antibodies was efficiently prevented by treatment with Xork-Fc. Combining Xork-Fc with ImmTOR tolerogenic nanoparticles has the potential to prevent de novo formation of anti-Xork antibodies and enable re-dosing of Xork.

734 Investigation of Hindlimb Clasping Following Intrathecal AAV9 Delivery

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With the growing advancements in AAV gene therapy and FDA-approved treatments, researchers are increasing their focus on possible adverse events following AAV delivery. Intrathecal delivery of AAV9 is often used to treat neurological and neuromuscular diseases. Despite promising therapeutic benefit, preclinical studies across different species have identified dorsal root ganglia (DRG) pathology following delivery of AAV9 by this route. Immune responses underlie DRG

toxicity, which may be caused by the capsid, transgene expression, or poor vector quality. Further studies are needed to understand this phenomenon. While DRG toxicity has been observed in non-human primates following AAV delivery, this has not yet been seen in mouse models. In this study, we aimed to investigate factors that may contribute to DRG toxicity: high viral doses, high transgene expression, and genome heterogeneity within manufacturing. In all studies, wildtype mice were treated with self-complementary AAV9 at post-natal day 10 via intrathecal lumbar puncture and were monitored weekly for weight, survival, and hindlimb clasping. At 8-weeks post-injection, tissues were harvested for quantification of transgene expression and histopathology assessments. To examine the effect of transgene expression, we administered vectors containing a weak or strong ubiquitous promoter to drive differential expression of the same transgene. To test the influence of genome size on DRG toxicity, mice were treated with vectors carrying the same promoter and transgene, but either 1.3kb or 2kb of DNA sequence between the inverted terminal repeats. A GFP coding sequence was added downstream of the transgene of interest in the 1.3kb construct to generate the 2kb construct, which is closer to the optimal packaging size of 2.4kb for self-complementary vectors. Across treatments or compared to vehicle, no differences in weight or survival were observed. Notably, progressive hindlimb clasping developed 4 weeks after administration in 60% of mice treated with the 1.3kb construct and was absent in mice treated with the 2kb vector. This behavior was independent of sex and litter and occurred in animals that were treated on different days. Hindlimb clasping has previously been associated with spinal cord atrophy in mouse models of ALS, however to our knowledge this behavior has not been directly associated with AAV. We did not find any relationship between transgene expression and hindlimb clasping, suggesting excess protein expression is not responsible. We hypothesize that the small genome size of 1.3kb resulted in packaging of DNA backbone past the ITRs, which was recently reported to cause adverse immune responses. Single molecule, real-time (SMRT) sequencing will be used to determine the DNA content that was packaged. Histopathological assessments of the DRG and additional organs are ongoing and will be reported. The findings from this study emphasize the importance of expression cassette design and size when developing AAV vectors for gene therapy and caution greater scrutiny of the packaged product.

735 Monocytes That Efferocytose Viable, but Not Heat Inactivated, MSCs Adopt a Distinct Immmunoregulatory Phenotype

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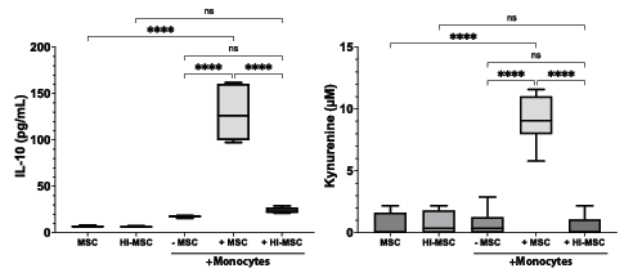
Intravenously infused MSCs are rapidly cleared from the body, yet a potent immunotherapeutic response is still observed. Recent work suggests that monocytes contribute to the clearance of MSCs via efferocytosis which begs the question of how variations in MSC health affect monocyte phenotypic response. We sought to characterize viable and HI-MSC efferocytic-licensed human monocytes by surface marker and soluble factor analysis to determine their phenotypic response. Human PBMCs and umbilical-cord MSCs were co-cultured for 24hr in complete culture media in polypropylene V-bottom microplates. To assess monocyte efferocytosis of MSCs, MSCs were stained with CellBrite Orange (CBO) membrane dye prior to co-culture. MSCs were either viable or heat-inactivated prior to the 24hr co-culture. Heat-inactivation was performed by 30-minute incubation of cells in a 50°C water bath. Following 24hr MSC:PBMC co-culture, monocytes were stained with fluorescent antibodies for CD14, CD16, CD86, CD163, and CD206 to assess changes in surface marker expression due to efferocytic-licensing by either viable or HI-MSCs. To assess monocyte soluble factor changes due to efferocytic-licensing with either viable or HI-MSCs, monocytes were isolated from PBMCs by negative selection prior to co-culture with MSCs. Viable MSC, HI-MSC, and monocyte only controls were plated as well. After the 24hr culture, all samples were re-plated to a polystyrene flat-bottom microplate with complete media supplemented with 200μM L-tryptophan. The samples were allowed to culture for an additional 2 days after which media was collected from all samples and assessed for IDO activity by kynurenine production and IL-10 by ELISA. All data was analyzed using GraphPad Prism 9 and all flow cytometry data was processed using FlowJo prior to statistical analysis. Surface marker analysis of monocytes following efferocytic-licensing with viable and HI-MSCs showed no significant difference in CD14 or CD16 expression compared to control. Expression of CD86 increased significantly in classical and intermediate monocytes efferocytically-licensed with viable MSCs while both viable and HI-MSC efferocytic-licensing resulted in significant CD86 expression of non-classical monocytes compared to control. CD163 expression significantly decreased for classical and intermediate monocytes that were efferocytically-licensed with viable MSC compared to control. Efferocytic-licensing of monocytes with viable MSCs, but not HI-MSCs, resulted in significant increases in IL-10 production and IDO activity compared to controls. Our work shows that efferocytic-licensing of human monocytes by MSCs results in distinct immunosuppressive phenotypic profiles depending on the health of the MSC utilized. The combination of increased CD86 expression, IL-10 production, and IDO activity with decreased CD163 expression is indicative of a potent immunosuppressive monocyte phenotype in response to efferocytosis of viable MSC. The combination of a slight increase in CD86 expression and no change in CD163 expression, IL-10 production, or IDO activity is indicative of a mild change in monocyte phenotype in response to efferocytosis of HI-MSC. This extends our understanding of MSC therapy by showing that efferocytosis of viable MSCs leads to immunosuppression by monocytes mediated by IL-10 and IDO.



Viable MSC efferocytic-licensed monocytes adopt an immunoregulatory phenotype. Monocytes cultured with viable MSCs for 24hr exhibit significant increases in IL-10 production and IDO activity compared to controls. HI-MSCs had no effect on IL-10 production or IDO activity.

736 Single Marker cMPL Enriches Long-Term Repopulating Human Hematopoietic Stem Cells

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Current gene therapy protocols for the treatment of inherited human blood disorders mostly rely on *ex vivo* genetic correction of mobilized peripheral blood (MPB) CD34+ hematopoietic stem and progenitor cells (HSPCs), a heterogenous cell population with predominantly lineage restricted progenitors and rare long-term repopulating hematopoietic stem cells (LTR-HSCs). Strategies to enrich LTR-HSCs within MPB CD34+ HSPCs could improve the efficiency of gene correction in these cells and enable broad clinical accessibility by reducing reagent cost. In humans, hematopoietic cells with a CD34+CD38-CD45RA-CD90+CD49f+ immunophenotype represent a population with the highest reported purity of LTR-HSCs. However, this subset remains heterogenous (Huntsman, Blood 2015) and the complex combination of markers precludes identification of LTR-HSCs in tissue sections. The hematopoietic cytokine thrombopoietin and its cognate receptor cMPL act as primary regulator of HSPC function. Surface expression of cMPL can enrich LTR-HSCs in mice, but its utility for purification of human LTR-HSCs has not been investigated. In this study, we sought to investigate whether cMPL can serve as a reliable and simpler marker to enrich LTR-HSCs in human adult CD34+ HSPCs. We first obtained MPB CD34+ cells from healthy volunteers and confirmed that surface cMPL expression measured by flow cytometry was significantly higher within the CD34+CD38-CD45RA-CD90+CD49f+ cell fraction relative to bulk CD34+ cells. To determine whether cMPL can enrich functional LTR-HSCs, we used fluorescence-activated cell sorting to partition human MPB CD34+ cells into $cMPL^{high}$ (top 10%) and $cMPL^{low}$ (bottom 10%) populations, and transplanted these cells into immunodeficient NBSGW mice. Serial peripheral blood (PB) sampling of primary xenografted mice revealed a striking difference in patterns of hematopoietic reconstitution over time between the two groups. In mice transplanted with $cMPL^{high}$ cells, human cell chimerism increased gradually throughout the 16-week engraftment period, while a progressive decline in engraftment was observed in the $cMPL^{low}$ group. Notably, mean human cell engraftment within the PB, bone marrow (BM) and spleen of mice transplanted with $cMPL^{high}$ cells was 209-fold ($p < 0.01$), 37-fold ($p < 0.0001$) and 283-fold ($p < 0.001$) higher than mean human cell chimerism in the $cMPL^{low}$ group at 16 weeks post-transplantation, respectively. These data suggest that $cMPL^{high}$ and $cMPL^{low}$ populations are distinctly enriched in LTR-HSCs and hematopoietic progenitors, respectively. Next, to quantitatively compare the frequency of self-renewing LTR-HSCs within the $cMPL^{high}$ and $cMPL^{low}$ HSPC subsets, human CD45+ cells obtained from the BM of primary mice were injected into secondary NBSGW mice at limiting dilution and BM engraftment was measured at 16 weeks post-transplantation (total period of engraftment: 32 weeks). After accounting for engraftment parameters (i.e., cell dose and engraftment levels) in both primary and secondary mice, extreme limiting dilution analysis computed LTR-HSC frequencies of 1 in 1,267 within the $cMPL^{high}$ CD34+ cell population, and 1 in 149,010 in the $cMPL^{low}$ CD34+ cell fraction, representing a 116-fold enrichment of LTR-HSCs using cMPL as a single marker purification strategy. Globally, our data suggest that cMPL effectively enriches LTR-HSCs in human adult CD34+ HSPCs. We foresee that a more stringent sorting scheme of $cMPL^{high}$ cells (e.g., top 1%) would enable further LTR-HSC enrichment that could approximate single-cell resolution. Additional studies are ongoing to evaluate the utility of a cMPL-based HSC purification strategy in the context of autologous HSC gene therapy.

737 Development of Short-Term and High-Efficiency Motor Neuron Induction and Enrichment Methods from iPS Cells Using Adenoviral Vectors

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Human pluripotent stem cells (hPSCs) are expected to be applied to the in vitro modeling of human intractable disorders and regenerative therapy. In particular, since neurons cannot be isolated from humans, induction of differentiation from human induced pluripotent stem cells (hiPSCs) into target neurons is highly useful for disease analysis and treatment of neurological disorders. Although rapid, efficient, and simple differentiation and induction methods using various inhibitors combination or viral vectors expressing differentiation-inducing factors have already been reported, problems such as contamination of undifferentiated cells still remain.In this study, we constructed two kinds of adenovirus vector (AdV) to induce differentiation of motor neurons from iPS cells, and showed rapid and efficient differentiation efficiency not only via Neurosphere but also direct induction method. Moreover, in order to remove the remaining undifferentiated cells, we examined the usefulness of AdV expressing a drug resistance gene. 201B7 cells and ChipSC18 cells were used as iPS cells. To demonstrate induction of motor neuron, we used ChAT for immunofluorescence and Hb9 for quantitation of mRNA levels.First, we compared the induction efficiency of motor neurons from iPS cells via Neurosphere, which is commonly performed. For this, we constructed the AdV (three-factor AdV) carried with Ngn2, Isl1 and Lhx3. After preparing Neurospere, we compared the motor neuron induction efficiency by the conventional inhibitor combination method (adding SHH, retinoic acid, and IGF-I) and the method of transducing with three-factor AdV. Three-factor AdV was confirmed to have significantly more ChAT-positive cells than the conventional method. In addition, the expression level of Hb9 was 7 times higher than that of the conventional method. In particular, many ChAT non-positive cells mixed and proliferated in the conventional method. We constructed the enrichment-AdV that specifically expresses the puromycin resistance gene in neurons and transduced it into mixed cells. We have successfully enriched motor neurons.Next, we examined whether motor neurons can be directly induced from iPS cells using miRNA-carried AdV (miRNA-AdV) that expresses miRNA in addition to the above three factors. With conventional methods, it took more than 3 weeks to induce motor neurons, but by using miRNA-AdV, ChAT-positive cells were detected in just 9 days from the start of induction. In 201B7 and ChipSC18 cells, ChipSC18 cells showed higher induction efficiency than 201B7 cells, especially in the direct induction method.These results suggest that this method is highly useful as a short-term, high-efficiency induction method for motor nerves. We believe that these methods can be expected to be applied to the safe treatment of diseases such as spinal cord injury.

738 Comparative Analysis of Gene Expression and Growth Factor Secretion in Stem Cells from Human Exfoliated Deciduous Teeth (SHED) and Other Tissue-Derived Mesenchymal Stem Cells; Therapeutic Potential with SHED for Neurological Diseases

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Stem cells from human exfoliated deciduous teeth (SHED) are mesenchymal stem cells (MSC) isolated from the dental pulp, the tissue derived from neural crest cells. SHED express surface MSC markers (positive for CD90, CD105 and CD73, negative for CD45, CD19, CD14, CD34 and HLA-DR), and have abilities of colony formation and trilineage differentiation same as other tissue-derived MSCs. To clarify differences between SHED and other MSCs, gene expression profiles of SHED were compared with those of MSCs derived from bone marrow (BM), adipose tissue (AT), and the third molar dental pulp (DP) on multiple donors. Principal component analysis and hierarchical cluster analysis showed that SHED have a distinct gene expression pattern from other MSCs and were classified in a different cluster. Gene ontology analysis revealed neurogenic, angiogenic, and cell migration-related genes were significantly more enriched in SHED than BM and AT MSCs, reflecting their neural crest derivation. Indeed, comparative cell culture studies demonstrated that SHED secreted some of neurotrophic, angiogenic, and migration factors more abundantly than other MSCs. Further, conditioned media with SHED enhanced the induction of anti-inflammatory M2-type macrophages from THP-1 monocytes more efficiently than those with other MSCs. Finally, we evaluated a therapeutic efficacy of SHED on a chronic stage of rat spinal cord injury model. Repetitive intravenous and intrathecal administration of SHED regenerated myelin sheath and improved a locomotor function. These results indicate that SHED possess high neuro-regenerative and anti-inflammatory capacities and should be a promising cell therapeutics for neurological diseases even at the chronic stage.

739 GNTI-122 is a Dual-Engineered Regulatory T Cell Therapy Product for Type 1 Diabetes with Enhanced Stability, Tissue Specificity, and Tunable IL-2 Signaling

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Regulatory T cells (Tregs) play a vital role in natural immune regulation and the prevention of autoimmune disease. Impaired function of Tregs can lead to autoimmune or autoinflammatory conditions. In Type 1 Diabetes (T1D), pancreatic islet-specific effector T cells (Teffs) cause inflammation and destruction of beta islet cells, leading to hyperglycemia. The introduction of islet-specific Tregs has the potential to control and reverse inflammation. Current sorted Treg therapies face 3 challenges, i) unstable phenotype, ii) a lack of tissue specificity, and iii) lack of IL-2 support. GNTI-122, an autologous cell therapy for T1D, is a genome-engineered Treg (EngTreg) designed to address these challenges by stabilizing FOXP3 expression, expression of an islet antigen-specific TCR, and a chemically inducible IL-2 signaling complex (CISC). GNTI-122 is engineered from bulk CD4+ cells with CRISPR-Cas9 to knock-in an MND promoter which bypasses TSDR-mediated silencing and stabilizes FOXP3 expression. This imparts a robust Treg phenotype (CD25, CTLA4, EOS, CD39, CD27, TNFRII, and low CD127 and CD70) in comparison to mock engineered cells, proving that engineering of stable FOXP3 expression enhances Treg phenotype. Upon stimulation, GNTI-122 maintains low expression of IL-2 and IFN-y, and increased expression of LAP and GARP, providing evidence of Treg function. The islet antigen-specific TCR, IGRP305-TCR, is engineered into the TRAC locus while simultaneously knocking-out endogenous TCRalpha and ensuring tissue specificity. Upon stimulation through its TCR by its cognate peptide, GNTI-122 cells display potent immunosuppressive activity in direct and bystander suppression assays. Importantly, in a polyclonal suppression assay, GNTI-122 suppresses activation and proliferation of a polyclonal pool of Teffs specific to islet antigens isolated from patients with T1D. Antigen specificity enables distinct activation of GNTI-122, allowing targeted immune regulation.The selective enrichment of the dual engineered cells is enabled by the addition of rapamycin-activated, CISCb into the FOXP3 locus, and the CISCg into the TRAC locus, providing IL-2-like signaling in only the dual engineered cells. Following gene editing, selective IL-2 signaling via CISC enables enrichment of GNTI-122 to >90% purity in culture. Activation of CISC in combination with TCR stimulation enhances engraftment and persistence in vivo where IL-2 is scarce in the inflamed microenvironment of the pancreas. Engraftment studies in NSG mice show rapamycin dose-dependent increase in engraftment and persistence, supporting the use of rapamycin in vivo. Altogether, GNTI-122 overcomes the key limitations of sorted Treg therapy, demonstrating direct, bystander, and polyclonal suppression in vitro, while maintaining IL-2 signaling support. Pharmacodynamic, pharmacokinetic, and pre-clinical safety data, including efficacy of murine surrogate models in vivo, support further evaluation of GNTI-122 in clinical trials.

740 Generation of PSC-Derived Class I MHC-Null, Antigen-Specific, Mature, Cytotoxic T Cells Using an Engineered *In Vitro* Organoid Microenvironment

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Cell-based immunotherapies in which autologous T cells have been engineered to express chimeric antigen receptors **(CARs)** and antigen-specific T cell receptors **(TCRs)** have produced impressive clinical

responses. Despite producing curative results, widespread application of adoptive cell therapies is limited by the complex and costly manufacturing processes for delivering patient-specific treatments, and the variability in T cell composition from each patient. To overcome these constraints, development of "off-the-shelf" engineered T cell products has garnered increasing interest. However, the requirement for an allogeneic source with this approach presents two main barriers: donor T cell mediated alloreactivity (causing graft versus host disease [**GvHD**]), and host-mediated rejection of HLA-mismatched allogeneic donor cells. While manipulation of these allogeneic mechanisms via gene editing of healthy donor peripheral blood T cells has been pursued, current techniques do not guarantee complete ablation of endogenous TCRs, and the gene-editing process carries the risk of genomic translocations and deleterious off-target events. Human pluripotent stem cells (**PSCs**) are a promising alternative allogeneic source as they are an infinitely self-renewing, and are amenable to gene-editing approaches and cloning to address alloreactivity and host-mediated rejection. Here, we report the development of Class I MHC-null, antigen-specific, naïve CD8 T cells from gene-edited PSCs using the "Artificial Thymic Organoid" (**ATO**) system previously developed by our lab, which induces highly efficient and reproducible differentiation of mature, naïve T cells from PSC sources. To prevent alloreactive TCR generation, we deleted both recombination activation genes (***RAG1*** and ***RAG2***) via CRISPR/Cas9 to generate *RAG1*$^{-/-}$*RAG2*$^{-/-}$ double knockout (**DKO**) PSCs. As predicted, T cell development from DKO PSCs was arrested due to the loss of endogenous TCRs. To support positive selection in the absence of endogenously rearranged TCRs, DKO PSCs were transduced with a lentivirus to constitutively express the HLA-A*0201-restricted 1G4 TCR recognizing the tumor antigen NYESO, generating DKO+TCR PSCs. Naïve, antigen-restricted T cells were exclusively generated from 1G4 TCR-transduced **DKO+TCR** PSCs that endogenously expressed HLA-A*0201, demonstrating that positive selection in the ATO system is restricted to the cognate MHC of the 1G4 TCR. Subsequently, DKO+TCR PSCs were edited to eliminate surface expression of Class I MHC by deleting Beta-2-microglobulin (***B2M***) via CRISPR/Cas9 to generate *RAG1*$^{-/-}$*RAG2*$^{-/-}$*B2M*$^{-/-}$ triple knockout (**TKO+TCR**) PSCs. Full T cell differentiation, including positive selection, was achieved from TKO+TCR PSCs by engineering the stromal component of the ATO system, MS5 bone marrow stromal cells expressing human Delta-like-ligand 4 (***DLL4***), to provide the proper signals for positive selection, including human HLA-A*0201, the 1G4 TCR's cognate MHC, as well as human *B2M*. Edited, TKO T cells exhibited potent antigen-specific cytotoxicity *in vitro* as well as tumor control *in vivo* that was superior to unedited T cells due to the absence of TCR mispairing, confirmed through 10X TCR sequencing. Overall, these results demonstrate for the first time that not only stem cells but also the *in vitro* microenvironment can be modified to deliver the necessary signals for T cell development when expression of genes involved in alloreactive mechanisms are disrupted.

741 Generation of Functional CD8ab T Cells from iPSCs in a TCR-Signal Independent Manner via Supra-Physiological Notch Signaling Provided by DLL4/VCAM-Coupled Microbeads

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The use of clonally-derived, induced pluripotent stem cells (iPSCs) as starting material for therapeutic T cell manufacturing would overcome many limitations of autologous Chimeric Antigen Receptor T cell (CAR-T) therapies. Complex, multi-stage genome engineering, large batch production and rigorous lot testing could provide a consistent source of off-the-shelf, functionally enhanced T cell products. However, this vision cannot be realized using existing T cell differentiation platforms, which fail to present Notch ligands with the precision and intensity required to control T cell differentiation in scalable, suspension culture. Moreover, current technologies do not allow researchers to explore the quantiative relationship between Notch signaling intensity and cell differentiation. We have previously shown that VCAM synergizes with DLL4 to enhance Notch signalling and progenitor T cell differentiation. We have extended the utility of this discovery through the creation of DLL4/VCAM-conjugated, magnetic microbeads ("Engineered Thymic Niche" technology), enabling precise and temporal control of Notch signaling in suspension culture. iPSC-derived CD34+ cells cultured with varying amounts of DLL4/VCAM1 beads demonstrate dose-responsive activation of Notch gene expression (e.g., Notch1, DTX1, TCF7), resultant T lineage commitment (e.g. CD5, CD7 expression) and differentiation (e.g. CD4, CD8 expression). Analysis of single cell transcriptomes during canonical T cell development in the thymus reveals that Notch signal intensity peaks in CD4-CD8- double-negative (DN) cells, then declines as cells transition to TCR-signal dependence during maturation from CD4+CD8+ (double positive; DP) to CD4-CD8+ (single positive; SP) cells. We queried whether supra-physiological, sustained Notch signaling could bypass the requirement for TCR signaling and intermediate differentiation stages toward cytotoxic T cells. We find that such conditions result in the rapid generation of CD8ab+ cells, bypassing the necessity of TCR/CD3 signaling during canonical differentiation. Using an iPSC cell line with a CAR knocked in at the *TRAC* locus (and thereby TCR-deficient), we apply this approach to generate CAR-expressing, CD8ab+ functional T cells, capable of multiple rounds of in vitro tumor cell lysis comparable to primary T cells.

742 Engineering a Gene Circuit-Enabled Cell Therapy with a Tamoxifen Regulated Safety Switch for Inducible Cell Death in Human Pluripotent Stem Cells and Their Derivatives

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Therapeutic cell products derived from human pluripotent stem cells (hPSCs) can be used to replace lost cells and therefore restore function and reverse disease for an array of clinically intractable conditions. While cell therapies have the potential to change the practice of medicine, all such "living" drugs carry potential risks. One safeguard against these risks is the implementation of a safety switch that can ablate transplanted cells from a patient if desired. We designed a novel gene circuit which functions as a Safety Switch regulated by tamoxifen, an FDA approved drug with extensive clinical history and ability to cross the blood-brain-barrier. The gene circuit-engineered Safety Switch is composed of a small molecule (SM) binding domain (ERT2) fused to Caspase-9 (Casp9) which dimerizes in the presence of tamoxifen metabolites, initiating the apoptotic pathway and cell death. To ensure the Safety Switch would be translatable to the clinic, we aimed to ensure the ERT2 SM binding domain would dimerize at concentrations of tamoxifen metabolites present in the brain at FDA-approved doses of tamoxifen. To address this, we computationally identified mutations within the SM binding region of ERT2 to build a large combinatorial mutant library. Screening of the combinatorial library produced hundreds of ERT2 mutants that were further evaluated for improved drug sensitivity in the context of synthetic transcription factor and activation of a reporter gene. Four representative engineered ERT2 mutants demonstrating improved sensitivity to tamoxifen metabolites (4-OHT and Endoxifen) were next tested in the context of the Safety Switch. All four engineered ERT2-Casp9 candidates showed greater than 95% killing efficiency after the addition of 1 μM 4-OHT, in HEK293T cells within 48 hours of induction. To maximize the expression of the Safety Switch gene circuit, we engineered it into hPSCs using the Sustained Transgene Expression Loci (STEL) platform for robust, stable, and ubiquitous expression of biological cargo. Briefly, the four engineered ERT2-Casp9 candidates were linked in-frame after the coding sequence of the endogenous *GAPDH* gene via a 2A peptide. Clonally derived *GAPDH::TamCasp9* hPSC lines demonstrated induced apoptosis after administration of the tamoxifen metabolites, 4-OHT and Endoxifen, at nanomolar concentrations. Here, we have demonstrated that hPSCs harboring a novel TamCasp9 gene circuit-engineered Safety Switch mechanism expressed robustly from the *GAPDH* locus can allow transplanted cells to be removed with pharmacologically relevant concentrations of tamoxifen metabolites.

743 Novel VLA-4 Inhibitors for HSPC Mobilization and Gene Therapy

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Background:Hematopoietic stem and progenitor cell (HSPC) mobilization regimens that are less toxic, more rapid and more potent than G-CSF and/or plerixafor are needed to improve transplant outcomes, reduce cost, shorten treatment times and facilitate gene therapy. We previously published that small molecule VLA-4 inhibitors (VLA-4i) induce rapid and reversible mobilization of murine HSPCs and are synergistic when combined with G-CSF, CXCR4 inhibitors (CXCR4i) and/or CXCR2 agonists. However, currently available VLA-4i don't have the appropriate combination of VLA-4 potency, aqueous solubility, bioavailability, and PK properties to be useful for HSPC mobilization. Here, we evaluated the efficacy of HSPC mobilization by novel VLA4i alone and in combination with CXCR4I for the purpose of gene therapy.Methods:Novel VLA-4i were synthesized and tested for potency using soluble VCAM-1 binding assays. VLA4i were then tested in mice and non-human primates (NHPs) alone or in combination with the CXCR4i plerixafor or BL-8040. HSPC mobilization was measured by flow cytometry (FACS) or Colony Forming Unit (CFU) assays. For competitive transplantation studies, mobilized CD45.1+ BALB/c mouse blood (10 uL) was injected into lethally irradiated CD45.2+ BALB/c recipients alongside $2.5x10^5$ CD45.2+ BALB/c BM cells (n=10/cohort). For the viral transduction experiment, mobilized CD45.1+ BALB/c HSPCs were transduced with lentivirus for GFP expression and injected into lethally irradiated CD45.2+ BALB/c recipients alongside CD45.2+ BM. HSPC engraftment and GFP transduction efficiency were monitored by FACS. Results:We developed novel VLA-4i by strategically attaching varied polyethylene glycol (PEG) chains to an optimized VLA4i binding core. Using mouse mobilization assays, we determined that a minimum PEG length of 24 units was required for extended HSPC mobilization. We then evaluated HSPC mobilization by WU-106 (VLA-4i with 24 PEG units) alone and in combination with plerixafor in NHPs. As expected, WU-106 alone was a weak mobilizing agent (**Fig. 1A**). Plerixafor alone was more potent and increased CD34+ counts 17-fold. The combination of plerixafor and WU-106 induced synergistic mobilization of HSPCs (50-100-fold; **Fig. 1A**). We next generated a longer PEG unit derivative of WU-106, named WU-125. WU-125 remained as potent as WU-106 at inhibiting VLA-4 and significantly extended murine HSPC mobilization both alone and when combined with plerixafor or BL-8040 (**Fig. 1B**). To explore if our VLA-4i + CXCR4i regimen represents an optimal graft source for gene therapy, we enriched murine HSPCs from the blood of mobilized mice by immunomagnetic selection, transduced them with GFP-containing lentivirus and injected them along with carrier BM into lethally irradiated congenic recipients. At 8 months post-transplant, we observed GFP+ cells among multiple lymphoid and myeloid cell lineages (**Fig. 1C**). This suggests that treatment with WU-106 + BL-8040mobilizes primitive HSPCs susceptible to gene therapy. Conclusion:We developed novel and potent VLA-4i that provide rapid and synergistic HSPC mobilization in combination with a CXCR4i. HSPCs mobilized with the combination of WU-106 + BL-8040 showed

efficient *ex vivo* viral transduction and stable long-term multilineage engraftment *in vivo*, which provides proof of concept that this approach could be used in gene editing therapy in the future.

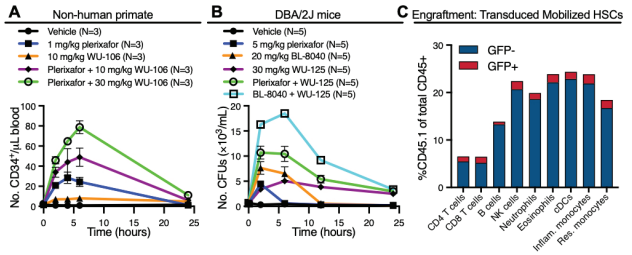

Fig. 1. HSPC mobilization and transduction. (**A**) NHPs were treated sequentially with the indicated regimens and the number of circulating CD34+ cells was determined over time by FACS. There was a 1-week washout period between each mobilization regimen. (**B**) Mice were treated as indicated and blood CFU numbers were determined. (**C**) Murine HSPCs harvested from the peripheral blood after treatment with WU-106 and BL-8040 were transduced ex vivo and transplanted into lethally irradiated congenic recipients. Engraftment of mobilized HSPCs was evaluated by flow cytometry 8 months after transplant.

744 Injectable Stem Cell Therapy Enabled by Microgels to Alleviate Discogenic Low Back Pain

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Chronic low back pain (LBP) affects over half a billion people worldwide and is commonly associated with intervertebral disc (IVD) degeneration. IVD regeneration remains an unmet clinical need, as current therapies such as surgical intervention and pain alleviation do not address the disease at its root cause - deterioration of the nucleus pulposus (NP) in inner core of the IVD. Notochordal cells (NCs) give rise to NP cells in development, have the potential to replenish NP cells and regenerate the IVD. NC are scarcely available in the adult but could be differentiated from induced pluripotent stem cells (iPSC). Our strategy is to develop an injectable cell therapy for LBP leveraging iPSC-derived NCs (iNCs) in combination with a cell delivery system based on hydrogel microparticles (microgels). The thermo-responsive hydrogels allow to encapsulate and precondition cells without introducing any cytotoxic crosslinker. The potency of this cell therapy was examined in vivo in a rat IVD degeneration model. The iPSCs were differentiated into iNCs using a previously published three-step process. The iNCs were encapsulated in fibrinogen-based thermo-responsive hydrogels in a microfluidic device at 10 million cells/ml gel. The microgels were purified to aqueous solutions for 7d (preconditioning). For the in vivo experiment, fluoroscopy-guided percutaneous needle injury was performed in rat lumbar discs (L4-5 & L5-6). After 2 weeks, iNC-loaded microgels were injected into the degenerated discs. The IVD height were evaluated using µCT and the discogenic pain was evaluated using biobehavioral tests. Our microfluidic device was designed and fabricated (Fig. 1A, B). The microgels have unified morphology and consistent cell density (Fig 1C, D). The diameter of the microgels was ~150µm in average after the purification (Fig 1E). Thermal gelation occurs around 21°C (Fig. 1F). The human cells encapsulated in microgel maintained comparable viability to the bulk hydrogel control (Fig. 1G). The 14d preconditioning of iNC-loaded microgels promoted ECM (Col2) deposition (Fig. 1H, I). In our needle-induced IVD degeneration rat model (Fig. 1J, K), the intradiscal injection of iNC-loaded microgels resulted in statistically higher cold hypersensitivity detected with acetone test (Fig 1L) and paw withdrawal pressure of rats compared to saline control (Fig. 1M), indicating less mechanical allodynia 2 weeks post treatment. The iNC-loaded group also showed increased IVD height at L4-L5 than saline control. The preconditioned iNC-loaded microgels showed positive effects in alleviating pain and regenerating IVDs as early as 2 weeks post-surgery. The high-quality injectable microgels and regenerative iNCs give hope for the clinical translation of minimally invasive cell therapies treating discogenic low back pain.

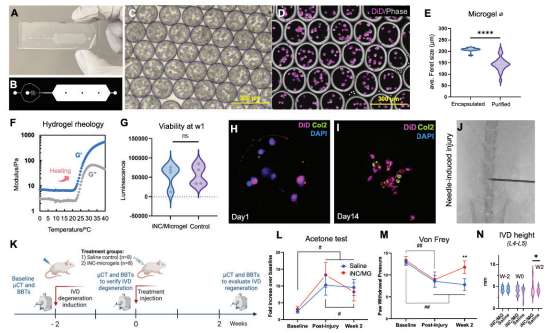

Fig. 1. Characterization and efficacy of iNC-loaded microgels as low back pain therapy. A, **B**. microfluidic device. **C**. Phase image of FF cell-loaded microgel in the device. **D.** Fluorescent image of DiD-labeled cells in FF microgels. **E.** microgel diameter calculated after encapsulation and after purification during which the oil is removed. **F**. Rheological property of FF microgel: storage modulus (G') and loss modulus (G'') changed with temperature heating ramp. **G**. Viability of microgel-encapsulated cells after 1 week pre-conditioning. Cells in bulk gel served as a control group. Immunostaining of DiD labeled iNCs with Col2 antibodies on Day 1 after encapsulation (**H**) and on day 14 (**I**), imaged by confocal microscopy. **J**. Posterior view of 21G needle used to induce disc degeneration percutaneously. **K**. Experimental design of the in vivo study. The iNC-microgels were compared to Saline control. **G**. Acetone cold sensitivity and **H.** von Frey biobehavioral tests indicating nociceptive behaviors (n=7; *p<0.05, **p<0.01 between groups, ##p<0.01 compared to baseline). **N**. Disc height quantification at baseline (W-2), 2 weeks post injury (W0) and 2 weeks post treatment (W2).

745 Discovery of Key Transcriptional Regulators of Alloantigen-Inducible Tregs Used for Cell Therapy

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Introduction: Graft-vs-host disease (GvHD) is the third leading cause of death in allogeneic hematopoietic stem cell transplantation (allo-HSCT). Unlike standard-of-care GvHD prophylaxis, alloantigen-specific (i.e., allospecific) type 1 regulatory T (Tr1) cells could prevent GvHD without impairing patient immunity against tumors and pathogens. Our allospecific Tr1 cells are already in two dose-escalating phase I/

II clinical trials, with promising early clinical results (ClinicalTrial.gov IDs NCT03198234 and NCT04640987). However, the current Tr1 manufacturing method results in a cell product with low Tr1 cell purity (≤10%), complicating dose escalation and data interpretation. We hypothesize that stable overexpression of a "master regulator" transcription factor (TF), which governs Tr1 cell differentiation, will result in a highly purified Tr1 cell product suitable for cell therapy. However, a master regulator TF(s) of human Tr1 cells is unknown. **Methods**: To identify the master regulator TF(s) of human Tr1 cells, we developed a functional epigenomics approach. We generated allospecific Tr1 cells (n=5) using the same protocol as is used for cGMP Tr1 production, then compared their epigenome (ATAC-seq) and transcriptome (RNA-seq) to 3 control T cell subsets. Differential TF binding site analysis, TF footprinting, and computational integration of Tr1 cell-specific epigenome and transcriptome to identify the domains of regulatory chromatin (known to be enriched for master regulator TFs) revealed six Tr1-specific TF candidates for the human Tr1 master regulator. The functional role of these candidate TFs in Tr1 cell differentiation was established by their CRISPR/Cas9-mediated knock-out (KO) or CRISPR-activation (CRISPRa) in parental CD4 T cells, which were then differentiated into allospecific Tr1 cell products. Finally, the Tr1 enrichment, phenotype, and function of TF-modified Tr1 products were compared to the wild-type controls. **Results**: Our functional epigenomics approach revealed new mechanistic insights into transcriptional regulation of human Tr1 cell differentiation and function. First, we established that EOMES (TF candidate #1), which is a proposed master regulator TF of allospecific murine Tr1 cells, plays no obvious role in the human allospecific Tr1 differentiation in our clinically-relevant model. Second, our CRISPRa experiments revealed that early activation of TF candidate #2, known to upregulate a key growth factor for human Tr1 differentiation, increases Tr1 cell frequency in allospecific Tr1 products by ≥ 50%. Finally, CRISPR KO of TF candidate #3 from parental CD4 T cells resulted in a significant reduction of Tr1 cell frequency in the allospecific Tr1 products, along with a significant reduction in the expression of co-inhibitory proteins LAG3 and CTLA-4. CTLA-4 is critical for the suppressive function of Tr1 cells; accordingly, Tr1 products without TF candidate #3 had a significantly diminished capacity to suppress effector T cell proliferation in response to alloantigen stimulation. **Conclusions**: We show that our functional epigenomics platform can be successfully applied to discover the function of human T cell TFs. We also show that TFs identified in murine Tr1 cells do not necessarily act the same in human Tr1 cells. Finally, we uncover two TFs that play key roles in differentiation and alloantigen-specific suppressive function of human Tr1 cells, which we will leverage to engineer new, purified Tr1 cell therapy.

746 A Study on Health Equity Challenges, Addressing Social Determinants of Health to Improve Access to Cell and Gene Therapies in US

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Introduction The evolving Cell and Gene Therapy (CGTx) marketplace is rapidly advancing, with multiple new therapies nearing commercialization or already commercially available. This rapid growth, however, sheds light on the multi-faceted, interconnected frictions within the current ecosystem that lead to CGTx patient access challenges, demonstrating how equity measures disparately affect access to CGTx. Through utilizing a framework that examines patient access to Zolgensma, an FDA approved Gene Therapy that treats Spinal Muscular Atrophy, the ways in which such equity measures affect patients in the CGTx community can be elucidated. We performed a waterfall analysis utilizing data from Komodo Prism, a database that covers 325M unique patient lives over 6+ year timeframes. We identified 1100 individuals within the population as individuals with SMA under the age of 2 years by filtering for SMA-specific ICD-10 diagnostic codes (Fig. 1). We then filtered this population by patients who underwent Zolgensma eligibility tests for AAV9 antibody levels, CVS, Creatinine, Troponin-I, and IFT (Fig. 1). The remaining patient population was then filtered to identify patients that received Zolgensma through J-code and CPT code filtering (Fig. 1). ResultsOf 1100 patients with SMA under 2 years of age, only 110 patients received Zolgensma representing a 90% decrease between eligible patients and patients that received therapy (Fig. 1). A 59% decrease of patient population was attributable to lack of confirmatory genetic testing leaving 451 patients remaining. From this population, a 47% decrease occurred when factoring in eligibility tests, leaving 238. Finally, of eligible patients, a 54% decrease occurred, and 110 patients of the original 1100 patient population received Zolgensma, representing 10% of the overall SMA patient population under 2 years of age (Fig. 1). DiscussionOur analysis demonstrates a significant decrease (90%) between patients in the SMA patient population under 2 years of age that are eligible to Zolgensma and the final patient population receiving the therapy. Through examining the different drop-off points within the waterfall analysis, it is likely that access and equability issues explain such a decrease and are especially troublesome for patients within the CGTx community. Patients that are originally identified as having Zolgensma face access issues in finding a timely diagnosis as mandatory newborn screening or public health services differs based on geographic region, socioeconomic status, and other demographic barriers. Furthermore, such a drop off can also be explained by lack of access to specialists that are qualified to diagnose and refer patients with rare conditions, and patients themselves face case navigation concerns while managing a complex qualification process for Zolgensma. Conclusion As the marketplace of CGTx evolves, innovative ways to improve equitable access must evolve alongside them. Individuals within the CGTx community such as knowledge leaders, manufacturers, insurance entities, and policy stakeholders need to determine unique and novel approaches to expanding access for all patients in the CGTx population.

747 Raw Material Variability in Biomanufacturing: A Case Study Using Poloxamer 188

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Gene therapy and cell-based products are growing rapidly and becoming a major part of the biotechnology industry. Meanwhile, this growth highlights several challenges in biomanufacturing, one of them being the raw material variability. This is a very critical bottleneck as it can lead to decreased productivity, process inconsistency and

compliance concerns. A critical material for cell shear protection, poloxamer 188, has a history of variability as the grades available in the marketplace had been optimized for other applications. BASF's poloxamer 188 product, Kolliphor® P188 Bio, is manufactured with the biomanufacturing industry in mind. To demonstrate minimal variability in and between batches, we tested samples of 2 batches of Kolliphor® P188 Bio in CHO-S and HEK293 cells. Samples from these lots were divided in 3 groups: early, middle and late samples depending on their drum number. We tested >>>1 samples of each designated type. Emulating a high shear environment, we recorded cell viability over 4 days of testing. Our data shows there is minimal change in overall cell performance between and within lots of Kolliphor® P188 Bio. Kolliphor® P188 Bio is a fit-for-function shear protectant offering shear protection while reducing variability in biomanufacturing processes.

748 Scalable Magnetic Bead-Based Cell Separation Technology for the Depletion of Receptor Positive Cells

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Allogenic therapies provide the promising possibility for off-the-shelf cell therapies, which could help to lower production costs and ultimately the therapy costs. However, this is accompanied by a large array of challenges with regards to their manufacturing, such as the need for large production scales to realize the commercialization for off-the-shelf therapies. With growing scales, the isolation methods need to be efficient, simple, universal applicable and should provide functional and safe cells to support this. The sorting of cells, based on magnetic beads is well-established, e.g. magnetic activated cell sorting. But with the small size of magnetic cell sorting beads used nowadays, being significantly smaller than a cell, this provides the risk of internalization, non-uniform magnetic labelling and low mobility due to low magnetization of the cell. Therefore, today's magnetic beads are limited in terms of large production potential.We have previously shown that our large (≈ 100 µm) magnetic bead system MAGicbeads is highly gentle towards cells (Brechmann et al., 2021) and provided excellent scalability at pilot-scale for the purification of mAbs (Brechmann et al., 2019). In the present study we have developed a new scalable separation process based on flexible Protein A base conjugated magnetic agarose-based beads for the isolation of receptor positive cell subpopulations. This system provides flexible adjustments of the receptor recognising antibodies, potentially targeting different receptors at different stages throughout the manufacturing process. Evaluation of the sorting capabilities of this novel flexible sorting system with a model population of $HER2^+$ cells and hMSCs showed high depletion efficiencies of up to 91 % (Brechmann et al., 2022). With high biocompatibility, high robustness against mechanical stress and minimal unspecific binding. This can provide an important insight for further large-scale application using high magnetised large particles for the purification of cells prior to the application to patients.

749 A Novel Chemically-Defined Medium Supports Superior Cross-Platform T Cell Expansion

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Lonza Walkersville Inc, Rockville, MD

One of the weak links in the current good manufacturing process of genetically engineered T cells for adoptive cell therapy is the use of human serum. Human serum and derived components are expensive, potentially contain adventitious agents requiring stringent safety testing and may contain factors detrimental for T cell expansion. In addition, the performance of human serum may vary considerably from lot to lot, necessitating screening and stockpiling. The supply of high-quality human serum may be insufficient to meet global demand in the near future as more blockbuster adoptive T cell therapies are approved and become one pillar for modern medicine. We have developed a cell culture medium, which incorporates only recombinant proteins, is free of undefined animal origin components, and requires only the addition of cytokines and activation agents, thus streamlining the CAR-T cell therapy manufacturing process. This medium supports superior T cell expansion compared to other commercially available T cell expansion media which require supplementation with human serum. T cell manufacturing processes utilizing this chemically-defined medium represent an important step forward in making adoptive T cell therapy more consistent and scalable to better serve patients. The absence of human serum and human plasma-derived proteins during the activation, transfection or transduction, and expansion processes necessitates some changes in common cell culture procedures. Alternative handling practices to be considered when using a chemically-defined medium for CAR-T cell therapies will be discussed.

750 Modulation of Single-Cell Gene Expression and Cell Function in Evolving Manufacturing Processes for Clinical Trials with Enhanced-Affinity T-Cell Receptor T-Cell Therapy Targeting the MAGE-A4 Antigen in Solid Tumors

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Introduction: Affinity-enhanced T-cell receptor (TCR) T-cell therapies targeting the intracellular cancer testis antigen MAGE-A4 have shown encouraging results in adults with advanced solid cancers.[1,2] Here we explore how TCR T-cell therapy manufacturing process evolution may modulate the function of ADP-A2M4CD8, the next generation counterpart of afamitresgene autoleucel (afami-cel; formerly ADP-A2M4), using single-cell RNA sequencing (scRNA-seq) and in vitro functional assessment. **Methods:** Autologous T-cell products were

manufactured from patient leukapheresis material by transduction of a self-inactivating lentiviral vector expressing either a MAGE-A4-specific TCR alone (afami-cel), or the same MAGE-A4-specific TCR plus an additional CD8α co-receptor (ADP-A2M4CD8), followed by ex vivo expansion. Afami-cel T-cells were expanded for 11-15 days in a Xuri bioreactor. ADP-A2M4CD8 T-cells were expanded for 10 days in a G-Rex bioreactor, with or without addition of an AKT inhibitor. Transduced CD8+ T-cells were isolated from retains of afami-cel and ADP-A2M4CD8 products and evaluated for differences in gene expression profile (GEP) at the single-cell resolution (ie, via scRNA-seq). Transduced CD4+ T-cells from ADP-A2M4CD8 products were also profiled as they demonstrate increased tumor cell lysis via the CD8α receptor. All subsets were assessed for their capacity to directly lyse tumor cells in vitro. **Results:** In unsupervised analyses, transcriptional profiles of ADP-A2M4CD8 showed considerable variation between clinical trial participants, for whom these products were manufactured, and between manufacturing processes. A notable shift from the afami-cel GEP was observed in ADP-A2M4CD8 T-cells, which were manufactured using G-rex based ex vivo expansion. A novel cellular subset, with an apparently less cytotoxic GEP, was seen in transduced T-cells from ADP-A2M4CD8 that was not seen in afami-cel. In addition to this novel subset, ADP-A2M4CD8 contained variable proportions of a CD8+ T-cell subtype prevalent in afami-cel products. This effect was enhanced by the inclusion of an AKT inhibitor during ex vivo expansion. These gene expression changes were accompanied by a delay in cytotoxicity with ultimately similar capacity to lyse target cells in vitro, which might suggest production of less differentiated cells. Translational correlation with the prevalence of these subsets will be discussed, along with the possible implications of these data related to the mechanism of action of afami-cel and ADP-A2M4CD8. **Conclusions:** Whole-transcriptome, single-cell investigation of the cellular subsets produced during manufacturing of afami-cel and ADP-A2M4CD8 indicate that infusion of cells with a less cytotoxic GEP does not prevent broad anti-tumor efficacy and may correspond with beneficial characteristics. 1. Van Tine BA, et al. Paper 61 presented at: CTOS 2022; Vancouver, BC, Canada. 2. Hong DS, et al. Ann Oncol. 2022;33 (suppl_7): S331.

751 Pre-Clinical Development of CT-1119, a Mesothelin-Targeting Chimeric Antigen Receptor Macrophage

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The immunosuppressive characteristics of the tumor microenvironment (TME) include the exclusion of T cells from the tumor and the surrounding stroma. Myeloid cells such as monocytes and macrophages, however, are actively recruited to the TME. Chimeric antigen macrophages (CAR-M) infiltrate the TME and exert targeted effector function, including phagocytosing tumor cells, activating the TME, and priming a broad anti-tumor adaptive immune response via T cell recruitment and antigen presentation. Previously, we developed CT-0508, a HER2-targeted CAR-M, which showed efficacy in a variety of pre-clinical models and is currently in a Phase I clinical trial for patients with HER2+ solid tumors. Mesothelin is a tumor associated antigen which is overexpressed in a wide variety of solid tumors, including lung, pancreatic, and ovarian cancers. CT-1119 was generated using the chimeric adenoviral vector Ad5f35 to express a CAR containing a fully human scFv targeting an epitope on the tumor associated antigen mesothelin. We evaluated its activity using both *in vitro* cell-based assays and *in vivo* xenograft tumor models. CT-1119 was highly viable with durable expression of the CAR. Importantly, the Ad5f35 vector yielded macrophages which were M1 (pro-inflammatory) polarized and resistant to re-polarization by M2 (pro-tumor) cytokines. Exposure to antigen amplified this effect with further down-regulation of M2 markers following stimulation. CT-1119 phagocytosed the A549 lung adenocarcinoma and MES-OV ovarian cystadenocarcinoma cell lines in an antigen-dependent manner and killed these cell lines *in vitro* in an antigen dependent manner. Engagement of the CAR yielded the release of pro-inflammatory cytokines such as TNFα in a dose-dependent manner in both cell-free and cell-based assays. CT-1119 significantly reduced tumor burden as measured by bioluminescent imaging in a murine xenograft model of lung cancer. In addition, Ad5f35 was utilized to generate functional anti-mesothelin CAR monocytes in a rapid single day process, yielding a population of myeloid progenitors restricted to M1-macrophage differentiation. In summary, these results show that the autologous human anti-mesothelin CAR-M are a targeted cell therapy capable of inducing a multi-modal anti-tumor mechanism of action.

752 Automated Perfusion-Based, Hollow-Fiber Bioreactor Supports Scalable T Cell Expansion

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The Quantum Cell Expansion System is a high-performing, automated platform for manufacturing both adherent and suspension cells. Quantum Flex is the next-generation device that features enhancements including a PC-based cell processing application used to create protocols, monitor runs in real-time, and manage a fleet of up to 100 devices. Quantum Flex also supports cell expansions on a small-scale bioreactor that can be used for process development and cell manufacturing from the low-quantity starting material. In this study, T cells were grown in both small and standard-size bioreactors utilizing different types of media. Both bioreactors support expansions with high viability, high yields, and desired phenotypes such as Tcm and Tscm while ensuring T cell exhaustion is minimal. New, non-DEHP disposables include media bags that have two ports, allowing for unique media handling strategies that reduce cost and reagent usage. Standard and small bioreactor expansions used less than 5 and 1.5 liters of complete media, respectively. Moreover, the small bioreactor can generate an autologous T cell dose of over 3B Cells with a starting seed of just 1M cells. The flexibility of Quantum Flex along with the small bioreactor allows scaling at any point of the manufacturing process while ensuring the base technology and expansion platform are kept consistent.

753 Spinner Flask Modelling of Wave-Style Bioreactors Enables Suspension-Phase Cell Culture Process Development of Allogeneic CAR-NK Cell Therapies

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Multiplex-engineered natural killer (NK) cells expressing therapeutic proteins are an exciting new modality in the cell therapy space with potential to be used as off-the-shelf allogeneic therapies for solid and hematologic malignancies. Clinical application of allogeneic NK cells requires a high-yield process that is most efficiently achieved using a suspension cell culture manufacturing platform; in the cell therapy field, wave-style bioreactors are widely used for this purpose. However, early process development of cell therapy manufacturing processes is commonly executed in static cultures, which can produce phenotypically and functionally distinct cells from suspension cultures. Static cultures are additionally limited from approaching suspension culture-specific variables, such as volumetric cell density control, and furthermore omit modeling of a static-suspension hybrid production process. ¶ As a result of these shortcomings, we developed a 150mL spinner flask format to better model a suspension cell culture environment. Two distinct CAR-NK products, CAT-179 and CAT-248, were produced in three different culture systems: 150mL spinner flasks, 1L static cultures and 2L wave-style bioreactors. Cells produced in spinner flasks showed comparable expansion profiles and transgene expression to wave-style bioreactors, while static cultures displayed increased cell yield but lower transgene expression, respectively. *In vitro* analysis of samples from the three culture systems revealed that cells produced in spinner flasks displayed a similar phenotype, cytotoxicity, and cytokine release level to those cells produced in wave-style bioreactors, compared to cells expanded in static flasks. ¶We next used the spinner flask model to optimize the timing of seeding a static co-culture of irradiated feeder cells (irrK562) and engineered NK cells into a wave bioreactor. We found that a static culture period of 4 days after irrK562 seeding was required to maximize cell yield, but the increased static culture period decreased expression of engineered transgenes. Use of the model revealed that the optimization of this parameter is critical to maximizing the yield of potent drug products in the CAT-248 process. ¶In summary, our data demonstrates proof of concept for the use of spinner flasks for culturing NK cells, providing a small-scale platform to model a wave-style suspension culture process more accurately. Use of this model will expedite scale-up process development as we pursue our clinical goals, shortening the path to clinical readiness and supplying a more complete data package in support of the clinical manufacturing of multiplex-engineered NK cells for solid tumor applications.

754 Bioadhesive Levan Based Nanofiber Using Coaxial Electrospinning Techniques for Cell-Based Tissue Engineering Therapies

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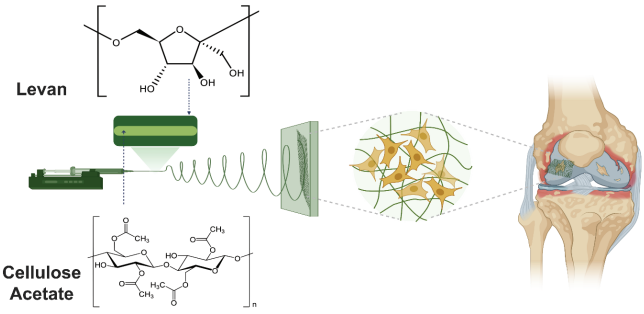
Electrospun nanofibers have been widely used for tissue engineering scaffolds because they can function as an extracellular matrix (ECM), which plays a significant role in cell proliferation, adhesion, and differentiation. Furthermore, they also enable modification to improve the functional properties of cells. However, the issues of weak mechanical strength and biological functions of electrospun nanofiber scaffolds hinder their range of practical applications. In this study, we established a uniform levan-based core shell nanofiber scaffolding using the co-axial electrospinning technique. The coaxial nanofiber matrices with levan in the shell and cellulose acetate (CA) in the core exhibited both highly reinforced mechanical properties and adhesive strength. Moreover, cells were observed to show improved attachment and proliferation on the bioadhesive composite nanofibers, particularly in terms of enhanced interaction with their structure during the early adhesion process. The biocompatible coaxial electrospun nanofibers also revealed suitable biodegradability properties and a lack of cytotoxic effects. This study also aimed to incorporate mesenchymal stem cells into nanofiber scaffolds fabricated by coaxial electrospinning for cell-based tissue engineering therapies. Bone marrow-derived mesenchymal stem cells (BM-MSCs) were cultured on levan-based nanofiberous architecture and we evaluated the osteogenic and chondrogenic differentiation ability of BM-MSCs *in vitro* using staining methods. Compared to the non-nanofiber scaffolds, BM-MSCs on nanofiber scaffolds exhibited enhanced osteogenesis and chondrogenesis by alizarin red staining and alcian blue staining, respectively. These results indicate that levan-based nanofiber architecture has the potential for delivering scaffolds to support BM-MSC's differentiability. Our results therefore suggest the application of the levan/CA composite nanofibers as an ECM in biomedical and tissue regenerative medicine applications.



755 Investigating Whole Blood as a Starting Material for CAR-T Cell Manufacturing

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Background: Chimeric antigen receptor T cells (CAR-T) have revolutionized the treatment of certain types of cancers. T cells are typically collected from patients via mononuclear cell apheresis, a long process that isolates designated cells while returning remaining blood components back to the patient. Those in need of T cell therapy have to undergo this process, and often have a central line catheter inserted into their chest/groin area when profound veins aren't present. This is typically more challenging and can be painful for pediatric patients. In contrast, whole blood collection is a routine process that can be done anywhere and does not require specialty apheresis collection centers. Our goal in investigating whole blood as a starting material for CAR-T manufacturing is to examine the phenotypic and functional characteristics of CAR T-cells manufactured in this manner, and determine if whole blood is a suitable alternative to apheresis. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via a closed-system, GMP compliant process using the Sepax (Cytiva). Whole blood samples were collected from eight healthy donors, including one donor who provided an apheresis and whole blood sample as a direct comparison. Isolated PBMCs were then were expanded and transduced with clinical-grade lentiviral vector encoding CD19/CD22 bispecific CAR using a fully-closed and GMP-compliant process, then cryopreserved on day 7. CAR-Ts were thawed and examined for phenotypic and functional properties. **RESULTS:** From a minimum of 29 mL of whole blood and 5-10x10^6starting CD3+ cells, we achieved a median day 7 T-cell yield of 105x10^6 (range 61 x10^6-188 x10^6) with 66% mean transduction efficiency, ultimately producing a median of 69.3 x10^6 transduced CAR-T cells (range 40.3 x10^6-124 x10^6). An ongoing clinical trial at the NIH Clinical Center utilizing the same CAR tested here (clinicaltrials.gov NCT03448393) has a safe and effective dose of 3x10^6 cells/ kg with an average patient weight of 60 kg. We show here that this therapeutic dose is attainable with a whole blood starting product given the scalability of the system and the fact that we can safely draw larger volumes of blood. Flow cytometry was performed to analyze the differentiation markers CCR7, CD45RA, and CD62L which showed T cell subtypes to be primarily mixed phenotype of CD45RA+CCR7- terminal effectors with high expression of CD62L, a marker of stem/central memory phenotype. High expression of CD25 and PD-1 (>50%) showed T cell activation with no more than 5-8% expression of exhaustion factor Lag3. CD19/22 CAR-T cells were incubated with GFP Nalm-6 ALL cells and Annexin V red apoptotic dye. We observed a rise in apoptosis with a simultaneous decrease in GFP Nalm-6 ALL fluorescence denoting tumor death, and targeted killing by CD19/22 CAR-T cells. Ongoing experiments are examining cytokine secretion using supernatants collected from CD19/22 transduced CAR-T cells co-cultured with Nalm-6 tumor cells to monitor levels of IFN-γ, TNF-α and IL-2. In conclusion, therapeutically relevant doses of CD19/CD22 CAR-T cells can be successfully manufactured from whole blood and we have shown that these cells exhibit high functional activity with targeted specificity.

756 Buoyant Microbubble-Based T Cell Activation Results in Significantly Reduced T Cell Exhaustion Profile

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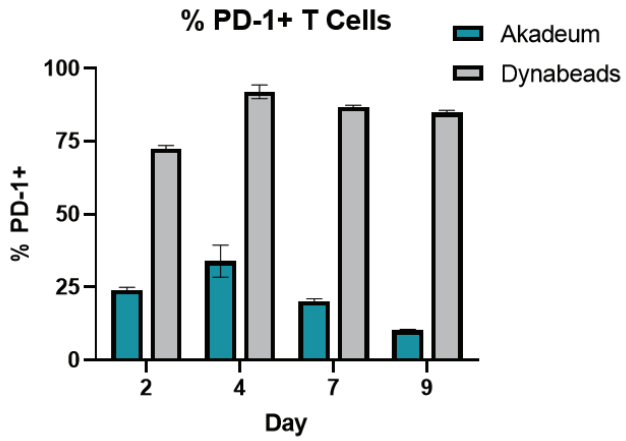
Advanced therapy medicinal products (TMPs) such as Chimeric antigen receptor T cell (CAR-T) therapy have proven clinical efficacy. The ever-increasing number of clinical programs demonstrates continued enthusiasm for these life-saving treatments. A critical early step in the manufacturing of CAR-T cells is the co-stimulation of T cells. Traditionally, the stimulation reagents utilized for the activation of T cells were soluble CD3 and CD28 antibodies paired with accessory cells such as antigen-presenting cells (APCs). The activation resulting from this method was often variable and dependent on the quality of cells used. More recently activation of T cells has been achieved using iron beads or iron nano-matrices coated with CD3 and CD28 antibodies. While these methods result in a high degree of activation and expansion, they force the T cells into prolonged and continuous stimulation which leads to a strong increase in exhaustion markers such as PD-1, LAG-3, and TIM-3. Since exhausted CAR-T cells may result in lower efficacy, we investigated an activation workflow using buoyancy activated cell sorting (BACS™) microbubbles. A process that combines selection, activation, and expansion into a single workflow powered by BACS microbubbles is presented in this study. Due to the natural buoyancy of the microbubbles, activation and expansion take place at the top surface of the culture vessel. As the stimulated T cells divide, daughter cells dissociate from the microbubbles and settle at the bottom surface of the culture vessel where they are no longer in contact with the activation signals. This separation from the activation signals prevents overstimulation and results in a 2-fold lower expression of exhaustion markers relative to the industry-standard activation methods - only 25-30% PD-1+ T cell when microbubbles are used compared to over 90% when iron particle-based methods are used. CD3/CD28 microbubbles reduce the expression of exhaustion markers without compromising T cell activation as evidenced by the rapid upregulation of CD25 and other standard activation markers. Additionally, buoyant activation using microbubbles yield enhanced effector T cell populations and industry standard expansion kinetics. Taken together, these data show that our microbubble-based workflow provides improvements to all of the critical aspects of CAR T cell generation while eliminating many of the most significant hurdles.



757 Process Development for Efficient CAR T Cell Manufacturing Using a Closed Instrument Workflow

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Over the last decade, researchers have demonstrated how (CAR) T cells can be effective in treating several devastating and often fatal cancers. CAR T cell therapies also show promising results for long-term antineoplastic effects, keeping patients in remission longer with the potential promise of a complete cure. Yet, current T cell therapy manufacturing processes are complex, manual, and labor-intensive. This impacts regulatory compliance and prolongs timelines to get therapies to patients. Furthermore, this could raise the price of CAR T cell therapy, limiting patient access to potentially life-saving treatment. A complete, closed, automated CAR T cell therapy workflow could improve consistency and safety of the CAR T cell therapies and reduce their costs. Our series of closed, modular instruments can be both physically and digitally integrated in a complete, automated, end-to-end CAR T cell manufacturing process. To do this efficiently, we have had to optimize the process for each of our modular instruments. Here, we show process optimization data for each of our modular instruments including a demonstration of how a closed, physical integration of two of our instruments—the Gibco™ CTS™ Rotea Counterflow Centrifugation System and the Gibco™ CTS™ Xenon Electroporation System—can lead to the efficient manufacturing of CAR T cells.

758 Defining T Cell Receptor Repertoires Using Nanovial-Based Affinity and Functional Screening

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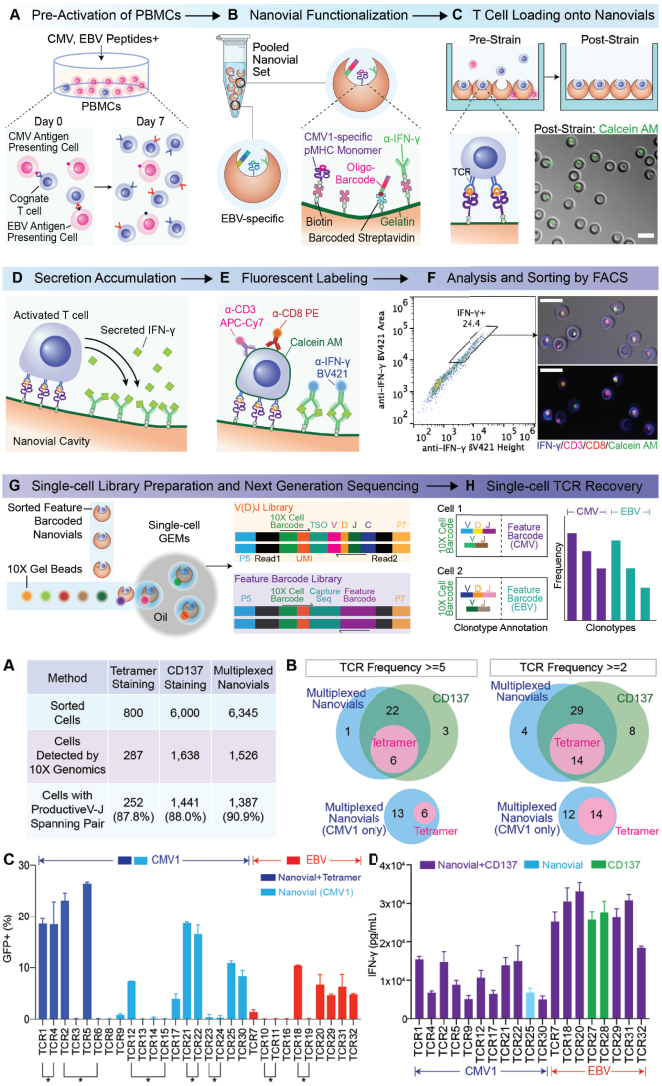
There have been encouraging successes in the use of engineered T cell-based therapies, including T cell receptor (TCR) immunotherapy, in treating cancer. However, one technical hurdle for developing effective TCR immunotherapy is to identify reactive TCRs from diverse sequence repertoires (10^8-10^{20}) that recognize targets with sufficient affinity and potency to induce apoptosis or activate other immune cells by secreting communication factors, such as cytokines. Current phenotyping approaches focus on affinity-based screening of differentially expressed surface receptors which fail to describe their functional potency or mimic the immune synapse of an antigen presenting cell. An ideal technology would combine antigen-specific enrichment and activation-based screening to achieve highly specific identification of functional TCR sequences along with knowledge of their cognate target epitopes. Here, we leverage cavity-containing hydrogel microparticles, called "nanovials", to achieve combined antigen-specific capture and activation-based high-throughput functional analysis and sorting of T cells based on secreted cytokines (Figure 1A). Each nanovial acts as both an artificial antigen-presenting cell, presenting millions of peptide-major histocompatibility complex (pMHC) molecules within the cavity creating a high avidity effect to capture and activate T cells even with low affinity TCRs, and as a capture site for secreted cytokines. Live cells on nanovials are sorted based on CD3 and CD8 expression and IFN-γ secretion, followed by single-cell sequencing to construct a TCR library with matching αβ-chains. In comparison to other screening approaches (MHC tetramer and CD137 staining), nanovials recovered the highest fraction of cells with a productive V-J spanning pair (90.9%) (Figure 2A). The nanovial workflow recovered significantly more clones (29) than gold standard tetramer staining (6) (Figure 2B). By labeling nanovials having different pMHCs with unique oligonucleotide-barcodes we could link TCR sequence to targets with 100% accuracy. A majority (78%) of clonotypes recovered using the nanovial technique were found to be reactive upon re-expression into NFAT-Jurkat GFP reporter cells and lead to functional secretion of IFN-γ when transduced into primary T cells (Figure 2C-D). Multi-parametric analysis of nanovials identified with high specificity an expanded repertoire of functional TCRs targeting viral antigens compared to standard techniques.



Method	Tetramer Staining	CD137 Staining	Multiplexed Nanovials
Sorted Cells	800	6,000	6,345
Cells Detected by 10X Genomics	287	1,638	1,526
Cells with ProductiveV-J Spanning Pair	252 (87.8%)	1,441 (88.0%)	1,387 (90.9%)

759 Engineering Therapeutic Cells through Rewiring of the miRNA Network

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To meet their full therapeutic potential, cell therapies need to overcome significant challenges relating to efficacy, safety, and cost. Clearing these hurdles requires increasingly complex cellular engineering. Gene editing approaches have provided much needed tools to genetically engineer therapeutic cells, but they mainly enable constitutive changes in gene expression with limited tuning capacity (e.g., gene KO). Gene silencing approaches based on RNA interference (RNAi) allow temporal and cell type-specific regulation of gene silencing activity, but current approaches such as synthetic siRNA or transgenic shRNA, are notoriously difficult to control. siRNA is intrinsically transient, and shRNA transgenes can be either epigenetically inactivated or result in supra-physiological expression and significant off-target effects. There is a need for new tools that can enable stable, tunable and programmable gene silencing with minimal or no off-target effects. We have established a new programmable gene silencing technology called GEiGS (Gene Editing-induced Gene Silencing) that overcomes many of the limitations of current approaches. GEiGS is a new way of leveraging the RNAi pathway in mammalian cells, by repurposing endogenous miRNA genes to express new target-specific siRNAs. Instead of inducing silencing through the delivery of synthetic oligos (siRNA) or through exogenous transgenes (shRNA), GEiGS works by introducing specific monoallelic gene edits into endogenous miRNA genes, re-coding their pre-miRNA (hairpin) region so that instead of the original physiological miRNA, they express a new siRNA directed against the target of interest. By exploiting redundancies in the miRNA network (editing individual members within families of related miRNAs) GEiGS can re-wire miRNA gene activity without detrimental loss-of-function effects. Repurposing endogenous miRNA genes as siRNA vectors has several significant advantages over existing gene silencing approaches: 1) minimal genome edits, no exogenous regulatory elements or transgenes; 2) stable and tunable silencing activity - endogenous miRNA genes have reproducible expression protected from epigenetic inactivation. Because the expression of the repurposed miRNA directly predicts the abundance (and hence activity) of the GEiGS siRNA, silencing can be dialled up or down through the choice of repurposed miRNA; 3) programmable silencing. Because miRNA expression patterns change in predictable manner during cellular differentiation or in response to changes in the cell state, these dynamic changes can be leveraged to program gene silencing to be induced 'in the right place, at the right time, and in the right cell type'; 4) specificity - gene silencing occurs with minimal or no off-target effects. We deployed GEiGS in human iPS cells to show it can specifically silence B2M, a component of the MHC-I complex and a target relevant for the generation of hypoimmunogenic cells for allogeneic cell therapies. We show that GEiGS is a very effective gene silencing approach that reproducibly allows stable homogenous silencing of MHC-I over a broad range of expression. By repurposing miRNAs with different levels of physiological expression, we were able to precisely tune MHC-I, ranging from near-complete silencing to distinct intermediate expression levels. Strikingly, GEiGS was able to maintain silencing even when MHC-I was induced by more than 10-fold following IFNg treatment. Importantly, whole transcriptomics analyses revealed that in contrast to siRNA and shRNA, which can show extensive off-target effects, GEiGS is very specific, showing no evident off-target effects associated either with the loss of the redirected miRNA genes or with silencing of unintended targets. This establishes GEiGS as a powerful new approach to gene silencing, and to our knowledge represents the first demonstration that endogenous human miRNA genes can be successfully re-wired to achieve specific regulation of new target genes.

760 Advancements in Manufacturing of CD8-Targeted Fusosomes Enhance Transduction of Resting T Cells *In Vitro* and *In Vivo*

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Sana is currently developing a CD8-targeted fusosome to potentially enable *in vivo* CAR-T therapy. Previously, we have demonstrated specific delivery of a CD19CAR transgene to CD8-positive T cells *in vivo* with a CD8-targeted fusosome based on an integrating lentiviral vector, which facilitates potent tumor killing in mice and B cell depletion in NHPs. Production of high potency fusosomes is important to deliver a sufficient dose in clinical trials in patients. Here, we describe end-to-end advancements, including improvements to the producer cell line, fusosome composition, and downstream processing, enabling improved potency and reduced impurities for CD8-targeted fusosomes. To establish a producer cell line for Sana's fusosome manufacturing platform, we adapted a cell line to serum-free and suspension growth, followed by single cell cloning. Suspension clones were screened for productivity of CD8-targeted fusosomes that carry a CD19CAR transgene. The top clones were further selected for growth and morphology. The most productive clones yielded up to 8-fold higher titer and improved growth relative to the control cell line. Our retargeted fusogen system is derived from a paramyxovirus family where two proteins, G and F, are required for particle attachment and fusion, respectively. Optimizing the G levels for CD8-targeted fusogen increased G density and proportion of complete particles (increase to 74% complete vs. 44% without optimized G levels). Utilizing the new producer cell clone and optimized G levels led to a 5-fold increase in functional titer per liter of culture and a doubling of infectivity for the crude material. Process advancements to the downstream clarification and filtration steps were also implemented to improve potency and purity of the final fusosome formulation. These changes increased the yield of active fusosomes 6-fold and process recoveries were doubled, yielding over 10 times more active fusosomes per batch compared to the initial process. The infectivity ratio improved 7-fold, while host cell protein and DNA were reduced by 10-fold and 1000-fold, respectively. The synergy of these updated downstream steps with producer cell and optimized G level advancements enabled formulations with over 50 times higher functional titers; potentially increasing doses delivered per manufacturing run and potentially lower dosing volume. CD8-targeted fusosomes with CD19CAR transgene made using these improvements in the manufacturing process significantly outperformed fusosomes made using the initial process with respect to transduction of resting T cells *in vitro* and *in vivo*. Critically, the specificity for CD8-positive cells was comparable or improved with fusosomes made with the improved process as compared to the initial process. In summary, we have shown that optimizing aspects of the manufacturing process for CD8-targeted fusosomes results in significantly higher titers, reduced impurities, and more potent delivery to resting T cells *in vitro* and *in vivo*.

761 Multiplex Cell Engineering of Next Generation Chimeric Antigen Receptor T Cells with Functional Silencing of Six Target Molecules

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Antion Biosciences, Geneva, Switzerland

Introduction: Chimeric antigen receptor (CAR) T-cell therapy is an established form of treatment for hematological malignancies. However, key challenges still remain, including a lack of efficacy against solid tumors and limited accessibility to these treatments. To overcome this, the need to multiplex engineer next generation CAR T-cells is essential, such that they can be provided as off-the-shelf (allogenic) therapies, capable of durable treatment response. The latter depends largely on the persistence of CAR T-cells, which not only relies on their fitness (continued functional activity), but also the ability to avoid rejection by the patient's immune system. We have developed an efficient strategy to create a next generation of CAR T-cells using a novel bimodal gene construct (miCAR) for simultaneous CAR expression and microRNA (miRNA) mediated gene silencing. Methods: Using a miCAR gene construct, we developed anti-CD19 CAR T-cells with multiplex silencing of six cell surface receptors, namely the T-cell receptor (TCR), human leukocyte antigen class I (HLA-I), CD52, PD1, TIM3 and TIGIT. Primary T-cells were modified via lentiviral vector transduction, expanded in G-Rex cell culture plates, and purified by depletion of TCR-expressing cells. Characterization of miCAR T-cells included flow cytometric immunophenotyping, functional activity against CD19-expressing tumor cells, and mixed lymphocyte reactions (MLRs) to demonstrate loss of alloreactivity and protection against allogeneic CD8 T-cells and NK cells. Results and Conclusions: We demonstrate robust and reliable production of CAR T-cells with >99% TCR negativity and efficient silencing of all targeted receptors, while maintaining a favorable immunophenotypic profile with 52-71% being of naïve memory phenotype (n=3 donors). Functionally, our multiplex engineered miCAR T-cells also maintained efficient and specific cytotoxicity of CD19-expressing cells. Finally, we show negligible alloreactivity in one-way MLR assays when miCAR T-cells were co-cultured with unmatched donor cells (n=3); and with "tuned" silencing of HLA-I (70 ± 10%), protection against both CD8 T-cell and NK cell mediated depletion (n=3 unmatched donors). We therefore show efficient multiplex engineering of functionally active, non-alloreactive and hypoimmunogenic CAR T-cells with silencing of six target genes.

762 Modular Semi-Automated Clinical Scale Manufacturing of CAR-T Cells for Cancer Immunotherapy

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With recent increases in clinical trials chimeric antigen receptor (CAR)-modified T cells have gained lot of enthusiasm in the field of cancer immunotherapy. Rapid development in this field and the need for generating cost effective, efficient cell therapies with predictable manufacturing results has also increased the demand for continuous manufacturing improvement. The conventional CAR-T cell manufacturing workflows involve labor-intensive steps with complex sequence of manual and open processes, with minimal to no automation. Introduction of automated modular system can reduce human interventions in the CAR-T manufacturing workflow. In the methods described here, we used a closed automated counterflow centrifugation platform, to automate several cell processing steps within the CAR-T cell manufacturing process including PBMC isolation from frozen leukopak, electroporation (EP) buffer exchange and final wash and concentrate. To achieve successful CART generation, we used Xenon™ Electroporation system to deliver the gene editing payload into activated T cells. First, we isolated the PBMC from frozen leukopak by using Rotea™ system and activated T cells using CD3/CD28 CTS™ Dynabeads™. The activated cells post de-beading was resuspended in gene editing buffer and then electroporated to deliver the Cas9 RNP complex and anti CD19 CAR using Xenon™ electroporation system. Post electroporation cells were expanded over 11 days to reach 4 x10^9 cells. Post expansion and editing efficiency analysis, we observed upto 60 % anti CD19 CAR knock-in efficiency. The CAR-T cells were finally washed and concentrated by Rotea™ and analyzed for transgene expression and then cryopreserved for further functional studies. The functional cancer cell cytotoxic assay against the CD19 presenting Nalm-6 cells showed effective killing by anti CD19 CAR-T cells compared to control T cells with no transgene payload. Utilization of closed automated workflows described here helps reduce the labor-intensive open system hurdles and generates functional engineered CART cells.

763 Towards a Consensus Platform for Last-Mile Cell Therapy Handling, Preparation, and Administration

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INTRODUCTION: Cell-based therapy is a novel treatment option in the toolkit of physicians. Given the success of CAR T cell therapies (CT), the field has exploded in interest and the FDA anticipates 5-10 new CT drug approvals per year over the next decade. Standards for cell collection and cell manufacturing have been established, yet standardized protocols for last-mile delivery of CTs (the process of handling, preparation, and therapy administration) in the clinic have yet to be established. Each FDA-approved CT product (e.g. Kymriah, Breyanzi, etc..) has a unique protocol. Hospitals are facing challenges with the overhead costs of CT; training and retaining of technicians, cell preparation, and post-infusion patient monitoring. With the upcoming deluge of CT drugs, there is a need for a better solution for delivering these therapies. Here we present a novel system developed to handle, prepare, and infuse CT products in an optimized manner. The system controls factors such as thaw rate, pressure, shear stress, wash, resuspension, and infusion, all to ensure cells are viable and functional once delivered into the body. Early data on several system capabilities and their impact on cell health is described hereafter. **METHODS & RESULTS:** The system enables rapid and controlled dry thaw in a pre-clinical setting in both cryobags and syringes (using unique freezable syringes developed for use with the system), matching ideal thaw rates recommended in literature. The system allows complex non-linear thawing, which has been shown to improve post-thaw cell health. The auto wash/resuspension capability has achieved levels of cell recovery on par with standard centrifugation based manual technique. The system can also filter and remove dead cells and debris, just prior to infusion. Given that cell debris is known to cause systemic toxicity in patients, this feature can reduce the severity of post-infusion toxicity. Finally, the controlled infusion capability significantly improves cell viability and functionality, in multiple cell types. The system and complementary disposable device can limit peak pressure, pressure fluctuations, shear stress, and cell-material interactions. The link between cell viability, functionality and therapeutic efficacy is well established in literature, with research demonstrating just 10-15% viability loss can completely negate the efficacy of a CT.

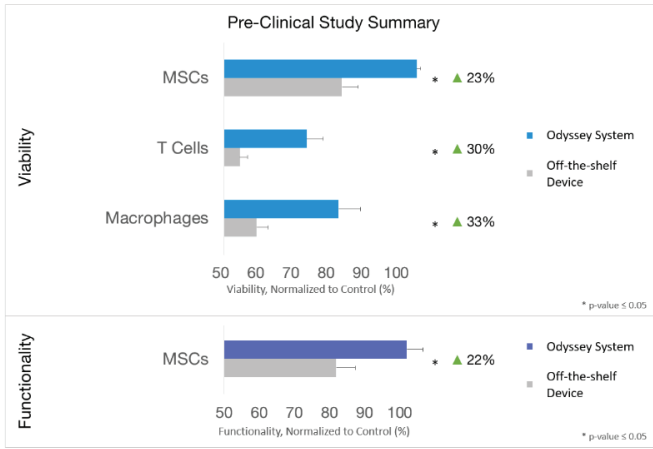

Figure: Results of pre-clinical studies using several cell types, including human MSCs, T Cells and macrophages. Cell viability was measured via an alamar blue assay, and functionality was measured via an ELISA assay of MSC IL-6 expression. n=3 per bar, * indicates $p<0.05$.

CONCLUSION: The handling, preparation, and final administration of CT products has largely been overlooked as an area for innovation and cost-reduction. The inconsistencies during patient-side CT delivery today include uncontrolled agitation causing cell death, breakage of the temperature preservation chain causing cells to senesce or apoptose, exposure to centrifugation or other separation techniques during washing and resuspension, and high pressure and shear stress during manual infusion. Cell-based medicines require strict control over their entire chain of custody. Here we describe a novel platform which enables standardization and improvement of last-mile CT delivery, a vital step for future clinical success.

764 Riboswitch-Regulated Chimeric Antigen Receptor, RiboCAR Enhances T Cell Activity

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Chimeric antigen receptor (CAR)-T cell therapy is a promising therapy against cancer. However, the level of CAR molecule is important for CAR-T cell activation and their anti-tumor activities. Here, we present the development of RiboCAR, a mammalian synthetic riboswitch-based gene regulation system for regulating CAR expression level via small molecule inducer. Unlike previously reported regulatable CAR platforms that utilize viral protease or chemical-induced protein dimerization, RiboCAR contains an ON riboswitch in the coding sequence of a CAR transgene, in which the aptamer functions as a sensor for a specific novel small molecule inducer. The expression level of the CAR gene with the riboswitch completely depends on the presence of the riboswitch inducer, with undetectable CAR in the absence of the small molecule and a dose response in CAR level reaching levels higher than constitutively active CAR upon maximal small molecule induction. The induced CAR expression diminished after withdrawal of the small molecule inducer. Further, CAR expression is titratable in response to the levels of the small molecule inducer. Consistent with small molecule induced expression of the CAR molecule, CAR triggered-activation of CAR-T cells is also controlled by the small molecule inducer. More importantly, T cells with RiboCAR showed enhanced target cell-stimulated T cell activation when compared with T cells constitutively expressing CAR. With a bioavailable small molecule inducer, CAR-T activity can be precisely tuned and "remotely" controlled *in vivo*, both temporally and spatially, thus improving the efficacy and providing a safety mechanism for CAR-T cell therapy.

765 Scalable Continuous-Flow Electroporation Platform That Enables Primary Immune Cell Engineering for Cellular Therapy Manufacturing

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Cellular therapies using autologous T cells expressing chimeric antigen receptors (CARs) have generated enthusiasm for their rates of remission but largely rely upon viral vectors for cellular reprogramming which are currently limited by expensive manufacturing, immunogenicity, and incompatibility with CRISPR/Cas9 gene editing. Electroporation has emerged as an alternative but widespread adoption requires addressing traditional electroporation difficulties including 1) variability, 2) arduous empirical optimization, and 3) limited throughput. In the present study, we describe a novel electroporation platform capable of rapid and reproducible electroporation that can seamlessly scale delivery from the research to the clinical scale. Our device consists of a continuous-flow, planar microfluidic channel (Figure 1A-B). Our design 1) ensures cells experience reproducible electric fields, 2) enables rapid sweeping of transfection parameters through robotic automation, and 3) seamlessly scales experimental throughput by scaling the channel width. As a demonstration of our system's capabilities, Jurkat or primary T cells from healthy donors were electroporated within CyteQuest's proprietary microfluidic flow chips. Jurkat cells were transfected with green fluorescent protein (GFP) encoded by plasmid DNA or mRNA. T cells were transfected four days after activation with CD3/CD28 antibodies with CRISPR/Cas9 ribonucleoprotein (RNP) complexes targeted against T cell receptor (TCR). GFP expression was measured via flow cytometry 24-h post-transfection. CRISPR/Cas9-mediated knockdown of protein expression was evaluated at 72-h post-transfection using anti-human TCR-α antibody and flow cytometry. Viability was measured during flow analysis using 7-AAD viability dye. To demonstrate seamless scaling, we transfected Jurkat cells with mRNA encoding GFP in either a 2- or 10-mm channel. Proportionally increasing the flow channel width (2 mm to 10 mm) with the volumetric flow rate results in identical mechanical, electrical, and chemical environments experienced by the cells. We observed roughly identical (less than 1% different) GFP expression and viability values in the 2- and 10-mm channel widths, demonstrating our ability to seamlessly scale (Figure 1C). To further demonstrate our scaling abilities, we transfected Jurkat cells with plasmid DNA encoding GFP in a 20-mm channel at a rate of 500 million cells/minute. Notably, we observed high GFP expression (86 ± 1.2%) with high viability (89 ± 1.4%) (Figure 1D). Finally, we delivered clinically significant cargo to primary T cells, using CRISPR/Cas9 RNPs to knock-down TCR expression. TCR expression was reduced from 89% ± 1% in control cells to 12% ± 2%, representing an ~88% knockdown (Figure 1E). These results demonstrate the capabilities of CyteQuest's novel electroporation platform for high efficiency and high viability delivery of multiple cargo to Jurkat and primary T cells. The innovations afforded by our microfluidic platform and its superior performance make it a promising system for non-viral cellular reprogramming for cell therapies.

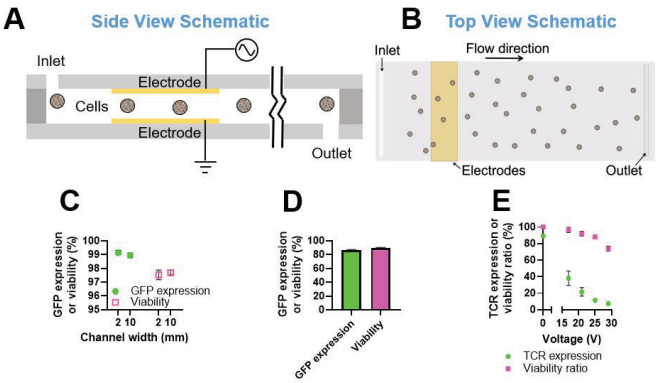

Figure 1: (A) Side and (B) Top view schematics of microfluidic flow chip. (C) GFP expression and viability data from Jurkat cells transfected with mRNA encoding GFP in either a 2- or 10-mm wide channel. (D) GFP expression and viability data from Jurkat cells transfected with plasmid DNA encoding GFP in a 20-mm wide channel. (E) TCR-α expression or viability data from T cells transfected with CRISPR/Cas9 RNPs.

766 Physiologix™ Serum-Replacement and NB ROC™ T Cell Culture Media Combination Shows Enhanced T-Cell Transduction Efficiency for CAR-T Therapy

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CD19 Chimeric Antigen Receptor (CAR-T) T cell therapy has shown a great potential for the treatment of B-cell malignancies and lymphoma and an increased number of studies are in progress for its translation to solid tumors. Success of this treatment is unfortunately limited by multiple factors such as donor-to-donor variability, patients response to the therapy and, manufacturing related issues. After leukapheresis, isolated T cells are manipulated under artificial conditions to be transfused back to the patient. Since these steps are conducted outside of the cell's natural surroundings, maintaining cell viability and their ability to proliferate without exhaustion and improving transduction efficiency are pillars for the success of the therapy. Thus, using the appropriate reagents is crucial for the treatment to be efficient, which will subsequently affect the quality of the end product and potentially increase the number of treated patients. Cell culture media is a critical factor to consider when improving the process as it is intimately linked to cell function and metabolism. In this study we tested a new cell culture media, NB-ROC™, developed specifically to support activated T cell proliferation and lentivirus transduction. Based on our previous data generated with the University of Pennsylvania on T cells, we supplemented NB ROC™ with 2% Physiologix™ XF (Human Growth Factor Concentrate (hGFC) , a cGMP, xeno-free media supplement made for stem cells and T cells). Compared to CTS™ OpTmizer™ T-Cell Expansion supplemented with 5% human serum, NB ROC™ shows on a Dynabeads-activated T cell model, a maintained proliferation rate and population doubling with a preserved phenotype, determined through the evaluation of surface expression markers such as PD-1, CCR-7 and CD45-RO over a period of 7 days. Lentivirus transduction data is more compelling as it shows an increased level by 20-30% compared to OpTmizer supplemented with human serum. Our data also confirms previous observations on Physiologix, as supplementing CTS™ OpTmizer™ with 2% Physiologix™ shows also significant transduction efficiency improvement compared to when it is supplemented with 5% human serum. All in all, our data show that NB ROC™; as serum free media combined with Physiologix™ ; offers a very promising alternative for an improved T cell transduction efficiency with a maintained phenotype and proliferation rate on both 6 well plate and G-Rex model. This set of data holds the promise for enhancing transduction efficiency for CAR-T therapy which will help ameliorate the process and bring more success to the field.

767 Development of an In-House Suspension Lentiviral Vector Production Cell Line to Accelerate Manufacture of Cell Therapy Products

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Lentiviral vectors offer excellent gene delivery vehicles for cell and gene therapy applications. Traditionally, adherent HEK293 T cells are transiently transfected with second or third generation vectors to generate lentiviral (LV) particles. Although this approach serves the need for preclinical research labs, adherent cells pose inherent difficulties when scaling up to produce GMP grade vectors. While production can be scaled up with adherent HEK293T using multi-stack cell culture flasks, it is not cost effective and operationally inefficient. Furthermore, dependency of FBS for growth poses additional disadvantage and safety risk for clinical products. To facilitate large scale production of GMP grade LV we adapted adherent HEK293T cells (ATCC, from SACF) to suspension culture using Thermofisher's serum free lentiviral production medium. The adapted 293 T suspension cells (293TS) produced lentiviral titers comparable or superior to Thermofisher's lentiviral production cells under different conditions. To build a self-sufficient inexpensive production platform, we optimized production conditions that eliminates the requirement of commercial transfection reagents or packaging cocktails. We validated this cell line for production of LV encoding chimeric antigen receptors (CAR). Currently, we offer high titer RUO grade LV production service using this cell line. Further optimization and scaling up is in progress.

768 Quantification of Single Cell Vector Copy Number in CAR T Cell Products Utilizing a Novel Microfluidic Technology

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Cell and gene therapies are altering the treatment landscape of intractable genetic disorders, including cancer and inherited diseases. One of the transformative solutions to treat cancer patients has been the Chimeric antigen receptor T-Cell (CAR-T) immunotherapy. As most CAR-T therapies rely on the modification of host cells with CAR using lentiviral vectors, followed by the re-introduction of the modified T-cell into the patient, the quality of these modified cells is extensively regulated to warrant safety. Accurate measurement of gene transfer as well as viral vector copy number (VCN) is critical to therapeutic development and a key attribute for assessing safety and efficacy. Conventional methods measuring VCN report out population average (bulk) or involve laborious and time-consuming single-cell clonal outgrowth procedures which could take up to months to complete. Here, we introduce a novel microfluidic workflow to precisely measure vector copy number as well as transduction efficiency across thousands of cells at a single-cell resolution in a shorter time frame. The technology is enabled by a two-droplet system. The first droplet encapsulates thousands of cells individually followed by lysis of the cells, releasing DNA from its heterochromatin state for uniform DNA interrogation. The second droplet generation involves uniquely barcoding individual lysates and amplifying the target DNA via multiplexed PCR inside each droplet. The targets are processed using NGS library preparation methods and the final products are sequenced on an NGS sequencing instrument. In this study, the single-cell VCN data generated using our vector-specific targeted assays was orthogonally validated by ddPCR single assays to obtain a correlation of 99% (Fig.1). Using vector-specific amplicons to classify cells as transduced or non-transduced allows us to calculate the percent transduction for each sample. Using admixtures of transduced and non-transduced cell lines, we show our % Transduction assay has >98.8% and >99.2% specificity and sensitivity.

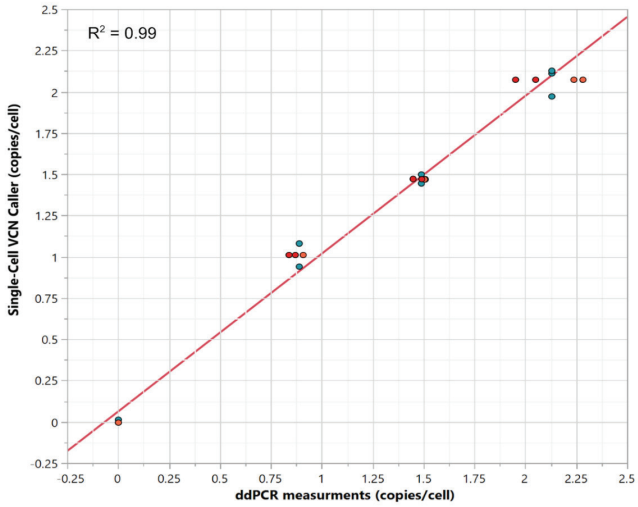

Fig.1: Additionally, using our single-cell DNA+Protein capabilities, we were able to genotype clonal Jurkat cells (VCN=2) and donor PBMCs based on the detection of SNV, and CNV, and phenotypically profile them based on surface protein marker expression (Fig.2)..

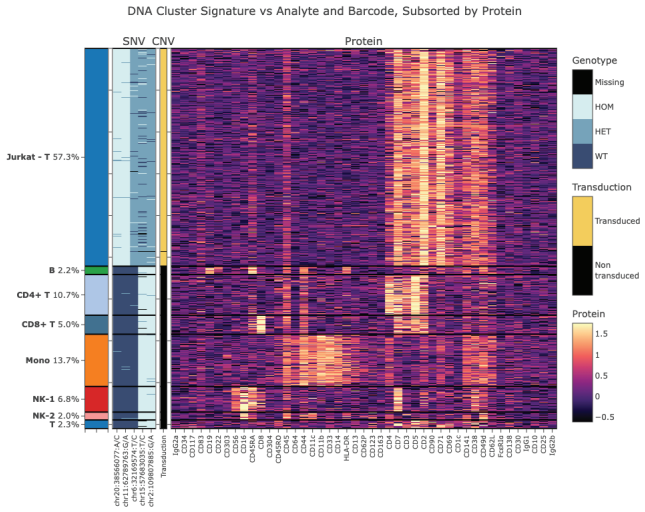

Fig 2: Future studies will evaluate the utility of this technology in measuring both CAR transgene copies and surface expression/ density of the CAR at the single cell level, improving the potential of our technology to provide more precise measurements of CAR T cell therapeutic product attributes before releasing the treatment to patients.

769 A New CAR Against the Intracellular Target MAGE-A4 is Capable of Lysing Multiple Myeloma Cells *In Vitro* and *In Vivo*

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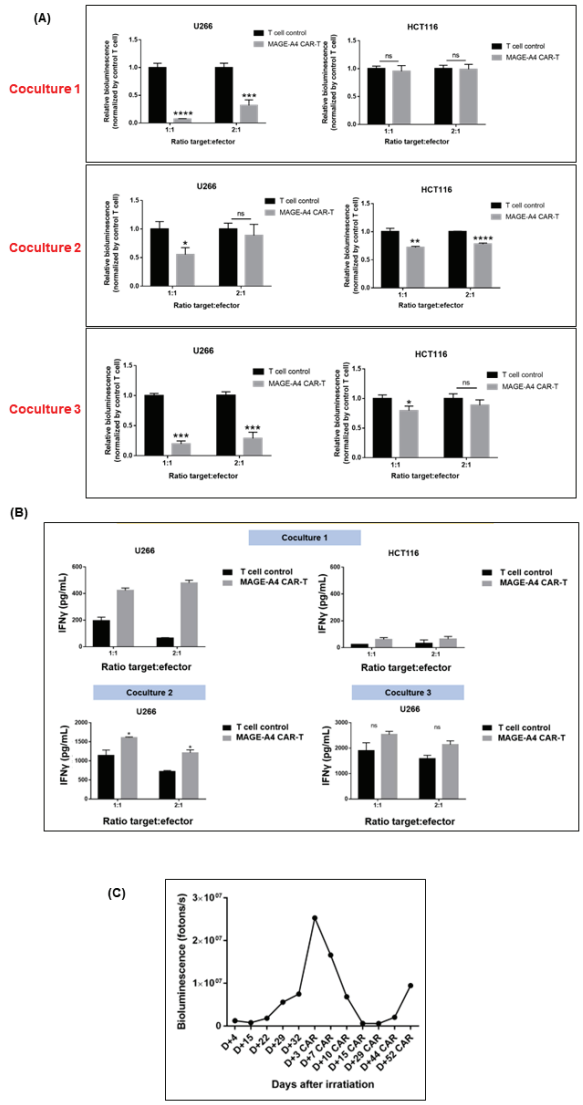
Background: Conventional chimeric antigen receptors (CARs) can recognize only cell-surface antigens in their native conformation. This greatly limits the number of suitable targets since most tumor-associated antigens are intracellular. The T cell receptors (TCR) expressed naturally in T cell usually have low binding affinity, and studies demonstrated that artificially increasing of TCR affinity might lead to unwanted binding to self-antigens. **Aims:** In this work, we report the development of CAR-T cells expressing a novel CAR capable of recognizing the intracellular tumor-associated antigen MAGE-A4 presented by HLA-A*02 molecules, which is the most frequent serotype in the western world. MAGE-A4 is a cancer-testis antigen expressed by a wide myriad of neoplasms, including late-stage multiple myeloma (MM). **Methods:** We generated a novel lentiviral vector coding a CAR harboring a recognition domain derived from an antibody specific to a MAGE-A4:HLA-A*02 complex. Next, we produced lentiviral particles and used them to transduce primary T cells. The resulting MAGE-A4/ CAR-T cells were cocultured with luciferase-expressing U266 MM cells (MAGE-A4$^+$) or HCT116 colon carcinoma cells (MAGE-A4$^-$) to assess their antineoplastic potential *in vitro*. We also evaluated IFNγ release during coculture and tested the antitumor potential in a MM xenograft model using NSG mice. **Results:** Transduction efficiencies of new MAGE-A4 CAR vector were high, reaching 57% to 84% in two transductions of different donor samples. These transduced cells displayed normal growth *in vitro* and stable expression of the CAR after 14 days of expansion. The percentage of in vitro tumor cell lysis using CAR-T cells derived from three different donors were 50%, 93% and 45%, respectively. Accordingly, CAR-T cells secreted IFNγ during coculture with U266^{luc} cells, but not with HCT116^{luc} cells, confirming the target-specificity. In an aggressive MM xenograft model, we observed that the MAGE-A4/CAR-T cells induced a strong tumor regression in established high-burden MM grafts, followed by relapse of the diseases after two weeks. **Conclusions:** We demonstrated the generation of a novel lentiviral vector coding a fully functional CAR which was able to redirect the cytotoxic activity of T cells against MAGE-A4+HLA-A*02$^+$ MM cells in vitro and in vivo. These results lay the groundwork for the establishment of a novel advanced cell therapy against MM and other MAGE-A4$^+$ malignancies.



770 Harnessing the Power of Multiomics from a Single Sample with Advanced Automation for Sample Handling and Processing

Andrea O'Hara[1], Laure Turner[2], Haythem Latif[1], Chris Mozdzierz[1], Ginger Zhou[3]

[1]Azenta Life Sciences, Burlington, MA,[2]Genewiz From Azenta Life Sciences, South Plainfield, NJ,[3]Genewiz, Inc./Brooks, South Plainfield, NJ

The omics era has greatly expanded the repertoire of approaches available for researchers and clinicians to unravel the complexity underpinning human health: Next Generation Sequencing (NGS) approaches can characterize genomes, epigenomes, transcriptomes and proteomes. The analyses are critical to assess in individuals both pre- and post-treatment during therapeutic development and early-stage clinical trials. Peripheral blood mononuclear cells (PBMCs) offer a window into the immune system that, when combined with these omics tools, can provide a near holistic view of immune processes across patient cohorts. Here we detail a workflow using a single blood draw to rapidly produce a diverse set of multiomics results including genomics, epigenomics, transcriptomics and proteomics. This starts with automated sample handling and processing of the primary blood draw to ensure high viability and yield of PBMCs, along with simultaneous plasma separation and collection. These samples are then aliquoted and simultaneously processed for automated and semi-automated whole exome sequencing, single cell RNA sequencing, epigenetic characterization and Olink proteomic assays. With this robust workflow and advanced robotics for sample handling and processing to minimize potential batch effects, all these datatypes can be produced within days of primary sample collection using minimal sample amounts. High throughput integrative omics workflows, as described here, drive greater insights in human health, allowing for a rapid combined approach to address the biological questions at hand.

771 Validation of a Powerful Analytical Method - ECLIA-Based Assay - for Total AAV Particles Quantification

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The analytical quantification of recombinant adeno-associated viral (rAAV) particles is an important part of the Quality Control panel for rAAV-based gene therapies. Correlated to viral genome quantification, the viral particles quantification allows the definition of empty/full particle ratio. This ratio is a critical quality attribute, as a high ratio can impact the therapeutic effect and increase rAAV immunogenicity. Viral particle quantification is performed both on in-process samples to define the critical process parameters leading to a better yield; and on the drug product to fulfill quality requirements. ELISA (enzyme-linked immunosorbent assay) is currently the gold-standard method for total AAV particle quantification within analytical departments. We recently developed an alternative ECLIA-based assay (ElectroChemiLuminescence Immunosorbent Assay) associated to the Meso QuickPlex SQ 120® plate reader. This alternative method shows a wider dynamic range than the reference method (4,5 log vs 2 log for the reference method). This larger range is a real asset to characterize both in-process and final drug product. Expanding the quantification range also reduces the number of retests in case of out of range sample (cost- and time-saving, improvement of sample comparability within the same run). Furthermore, this ECLIA-based assay presents a lower cost than the reference method (75% saved). Reducing the quality control cost is part of the current challenges in gene therapy, in order to reduce global manufacturing cost and improve accessibility of these therapies to patients. To better integrate the industrialization rAAV work flow, this ECLIA-based assay was validated, as required by the good manufacturing practices (GMP), for the quantification of total rAAV8 viral particles using the EMEA and FDA guidelines for bioanalytical method validation. The validation showed a broad quantification range, from 1,25E7 particles/mL to 2,50E11 particles/mL. The specificity and selectivity performances demonstrated the absence of interference from standard culture media of HEK293 (human embryonic kidney 293) and Sf9 (clonal isolate of Insect Spodoptera frugiperda Sf2) cells, as well as the absence of cross-reactivity with rAAV serotype 2. The between-run and within-run precisions showed a coefficient of variation below 20% and 36%, respectively. Finally, the accuracy performances (below

2-fold from target concentration) were comparable to those historically obtained between-run with the reference method and were aligned with internal validation performances for viral genome and residual DNA quantification by qPCR. In conclusion, this alternative ECLIA-based assay was successfully validated for routine use within the production workflow of our AAV vector core and could be included in a global tech transfer to a GMP environment.

772 Method Qualification of Mass Photometry for the Determination of Full-to-Empty Capsid Ratio for rAAVs Using the Refeyn System

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Packaging heterogeneity in recombinant adeno-associated virus (rAAV) manufacturing leads to potential product-related impurities including 'empty' capsids, capsids containing partial vector genomes, and capsids containing host cell or plasmid-derived DNA impurities. Methods for calculating full-to-empty ratios often place capsids into two broad categories: capsids containing DNA of approximately the size of the full-length vector ('full') and capsids which contain little to no DNA ('empty'). The ability to characterize capsids that contain shorter DNA fragments or fragments that are substantially longer than the full-length vector depends on the method used and the length of the vector genome. The full-to-empty ratio of rAAV capsids is considered a critical quality attribute because it may affect both safety and efficacy. The total capsid load of both 'full' and 'empty' capsids may contribute to immunogenicity and acute toxicity, which has been observed in clinical trials with high doses. On the other hand, there is evidence that empty capsids can reduce clearance by acting as decoys, resulting in increased potency *in vivo*. Therefore, the full-to-empty ratio should be carefully considered during development. Manufacturing processes should deliver consistent full-to-empty ratios, and methods to monitor this attribute are required for release of drug product. Analytical ultracentrifugation is the current gold standard method for determination of full-to-empty ratio. Although this method provides accurate and precise results, significant sample volume is required, and analysis must be carried out by highly skilled operators. Alternative methods, such as cryogenic electron microscopy (CryoEM) require less volume, but also need expensive specialist equipment and skilled operators. Recently, mass photometry has emerged as an alternative method for the determination of the full-to-empty ratio. This method provides results in minutes with microliters of sample using a benchtop instrument (Refeyn) and requires only basic operator training. To demonstrate that the method is fit-for-purpose in monitoring the full-to-empty ratio of rAAV samples, we performed method qualification according to the principles of ICH Q2(R1) using rAAV samples in matrices and buffers commonly used in the production processes. Here we present data that support future validation of the method for use as a release assay in a regulated environment.

773 Utilizing AAV Entry Factors & Small Molecules to Synergistically Enhance Vector Transduction and Transgene Expression to Facilitate the Development of *In Vitro* Cell-Based Assays for AAV Gene Therapy

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Translational Medicine, Asklepios BioPharmaceutical, Durham, NC

A common impediment to the development of *in vitro* cell-based assays for Adeno - associated virus (AAV) gene therapies (such as potency and AAV NAb assays) is poor transduction and transgene expression, which prevails across cell lines and AAV serotypes. Recently, several proposed AAV entry factors have been identified, whose overexpression could enhance AAV transduction efficiency. In this study, we tested a panel of proposed AAV entry factors in HepG2 cells by transient overexpression combined with AAV8 transduction. Overexpression of the AAV receptor (*AAVR*) prior to AAV8 transduction resulted in a 19-fold enhancement of transduction, whereas G-protein coupled receptor 108 (*GPR108*) expression increased transduction by 2-fold, and laminin receptor 1 (*LAMR1*) and transmembrane 9 superfamily member 2 (*TM9SF2*) expression had no effect compared to control. *AAVR* overexpression was further tested in an alternate cell line, HEK293, with a different serotype, AAV2i8, and a similar enhancement of transduction efficiency was observed. Next, a panel of small molecules were screened in HepG2 cells for their ability to enhance transgene expression. Twenty-four hour treatment with 10 μM of the topoisomerase II inhibitors, etoposide and teniposide, improved transduction efficiency 3-fold and 2.4 μM of the ER-associated protein degradation (ERAD) inhibitor, Eeyarestatin 1, improved transduction 4-fold compared to non-treated. Finally, the small molecules and AAVR entry factors were evaluated in combination for a synergistic effect. Overexpression of *AAVR* and 10uM etoposide treatment resulted in a 31-fold enhancement of transduction over control, confirming the synergistic effect between *AAVR* and small molecule treatments, suggesting that they impact different steps of the AAV transduction and transgene protein expression process. We believe the approach could be broadly applied to a range of cell lines and AAV serotypes to enable more rapid development of *in vitro* cell-based assays for AAV gene therapeutics.

774 Measuring RAAV Genomic Titer, Viral Particle Titer and Full/Empty Capsid Ratio on the Applied BioSystemsTM QuantStudioTM Absolute QTM dPCR System

Kimberly Lim
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Recombinant adeno-associated viruses (rAAV) are widely used for human gene and cell therapies due to their low immunogenicity, vast tropism and efficient and persistent gene transfer properties. However, effective potential dosing requires the use of well-characterized and optimized concentrations of viral vectors. Accurate and consistent viral titer measurements are needed throughout all phases of rAAV vector production from early stages of developing AAV expression systems to production scale-up and

final product release to ensure precise and appropriate dosing. Quantification of genomic titer (GC/mL) and viral particle titer (vp/mL) as well as the ratio of full to empty capsids are important rAAV attributes. However, quantification is not done in a harmonized manner. Other limited methods can measure single rAAV characteristics: qPCR measures genomic titer while viral particle titer is measured by ELISA. Full/empty ratio can be inferred from the combination of qPCR and ELISA data or measured independently by electron microscopy, analytical ultracentrifugation, or high-pressure liquid chromatography. We have demonstrated the feasibility of measuring genomic titer, particle titer and the full/empty ratio using a combination of proximity ligation assay (PLA) technology and Taqman assays on a qPCR platform. However, this approach requires the use of standard curves for determining particle and genome titers. As such, the method is more susceptible to inter-assay variation. Here, our proof-of-concept experiments demonstrate that genome titer, viral particle titer and full/empty capsid ratio can be measured on the Absolute Q platform using PLA and Taqman assay workflows (Figure 1). Digital PCR offers a simplistic approach to measuring nucleic and protein concentrations without the need for a standard curve. For two AAV serotypes tested, the full/empty capsid ratio obtained from dPCR aligned closely with the same measurement derived from qPCR standard curves. We also observed that the estimated viral particle and genomic titers were higher in qPCR compared to dPCR; this over-estimation by qPCR titers may be due to the reliance on a standard curve generated from a reference sample of the same serotype(s). In summary, we demonstrate the initial feasibility of determining AAV genome titer, viral particle titer and full/empty capsid ratio in a single duplexed dPCR. In this work, we used commercially available purified AAV particles as the test samples. Further work is needed to understand the impact of using unpurified or partially purified samples on duplex assay performance. Similarly, the impact of different purification methods on performance must be investigated. Overall, this idea overcomes several important challenges in AAV characterization: 1) the need for separate analytical platforms for genome and particle titer measures, 2) the need for standard curves for quantification, and 3) the need for fast and consistent results.

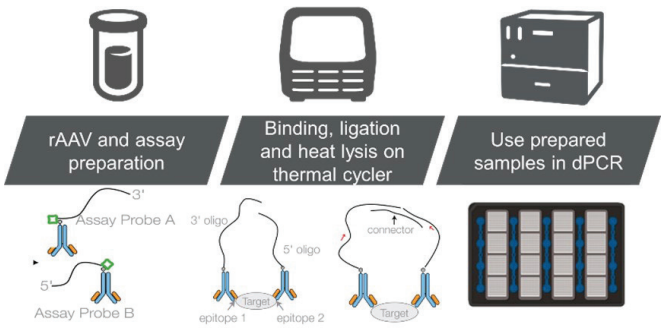

Figure 1. AAV-PLA workflow

775 A New Two-Dimensional Droplet Digital PCR Method for AAV Intact Viral Genome Titer Analysis

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Recombinant adeno-associated virus (rAAV) has been a vector of choice for *in vivo* gene delivery with many clinical trials for the treatment of human diseases. The clinical use of rAAV in gene therapy requires a precise quantification method for viral vector products. The current single channel ddPCR method provides incomplete pictures regarding rAAV genome integrity, i.e. complete vs. partial genome. In this study, we applied multiplexing 2D ddPCR to thoroughly characterize a rAAV product by providing new information on intact viral genome titer. Particularly, we designed the amplicon sites targeting both ends of the viral genome to ensure capturing the full-length viral genome in the analysis. We showed that the selections of appropriate PCR amplification sites are critical to precisely evaluate the viral genome integrity. Together with the application of linkage analysis using QuantaSoft software, we can accurately calculate the intact viral genome titer and the percentage of full-length rAAV among all genome-containing species in the product. Therefore, this new method can serve as quick characterization and titer assays for both process development and final product release with improved robustness, accuracy and precision.

776 Allometric-Like Scaling of AAV Gene Therapy for Systemic Protein Delivery

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Introduction: The use of adeno-associated virus (AAV) as a gene delivery vehicle for secreted peptide therapeutics can enable a new approach to durably manage chronic protein insufficiencies in patients. Yet, dosing of AAVs has been largely empirical to date. In this study, we explored the dose-response relationship of AAVs encoding a secreted Gaussia luciferase (GLuc) reporter or a bioactive candidate (parathyroid hormone; PTH) to establish a mathematical model that can be used to predict *in vivo* steady-state protein concentrations in mice based on steady-state secretion rates *in vitro*. **Methods:** A pharmacokinetic study was performed following intravenous administration of AAV2-EF1a-GLuc at two doses ($2.5x10^9$ and $1x10^{10}$ vg) in mice. GLuc concentration was measured in the plasma for up to 8 weeks post-injection. Tissue-specific vg content was measured by qPCR to identify target tissues. *In vitro* secretion assays were performed by transducing HepG2 cells with AAV doses ranging from 10^3 to 10^7 vg. A continuous perfusion bioreactor culture system was used to measure GLuc secretion rate *in vitro* as a model for tissue release in the bloodstream. Dose-response data were plotted for both systems and fit to a E_{max} model using GraphPad Prism. AAV2-EF1a-PTH vectors were injected in mice at two doses: $10^{10.8}$ or $10^{11.3}$ vg. For model validation studies, PTH steady-state concentration was measured in the plasma at weeks 3 and 5 post-injection. **Results:** The level of secreted luciferase reporter *in vivo* reached a steady-state concentration (C_{pss}) 3 weeks post AAV injections (Fig.

1A). The concentration of vg in liver tissues was over 10 times higher than other tissues, being considered the primary organ of protein production (Fig. 1B). The dose-response curves were well-fit by a E_{max} model, with a maximum steady-state secretion rate (R_{ss}) of 46.35ng/h (Fig. 1C). An *in vitro* dose-response curve was then derived as a model for liver parenchymal cell transduction, using the same AAV2 vector. A similar hyperbolic relationship was found between viral dose and R_{ss} *in vitro* (Fig. 1D). Parameter values for both systems were compared and a scaling equation was generated (Fig. 1E). The predictivity of our scaling model was confirmed using an AAV2 vector encoding a therapeutic protein. Predicted concentrations of PTH were calculated as a function of dose, considering PTH-specific parameters. Predicted values were very close to actual data from animals receiving two different doses of the vector (sensitivity: ± 4 pg/mL) (Fig. 1F). These results support the applicability of these scaling factors for other secreted transgenes delivered by AAV2. **Conclusions:** These studies provide a framework for scaling of *in vitro* studies of AAV-driven secreted proteins to target specific *in vivo*blood concentrations. Our model confirmed that plasma steady-state concentrations of secreted proteins expressed by AAVs can be guided by *in vitro* kinetic secretion data laying groundwork for future customization and model-informed dose justification for AAV candidates.

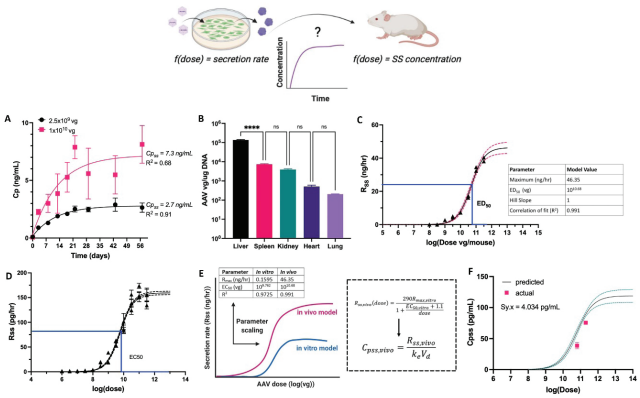

Figure 1. Developing a model for predictive dosing of gene therapeutics expressing secreted proteins. (A) Plasma concentrations of GLuc over time following AAV injection. (B) Viral genome in various tissues. (C) Dose response of steady-state rate *in vivo* with extracted model parameters. (D) Calculated *in vitro* secretion rate as a function of viral dose. (E) Parameters from both systems can be used to derive a scaling relationship. (F) Equation from model derivation can be used to predict concentration of PTH as a function of dose *in vivo*. Pink data points show actual concentrations.

777 Single Molecule, Real-Time Sequencing of Replication-Competent Adeno-Associated Viruses in Gene Therapy Vector Preparations

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Adeno-associated virus (AAV) vectors continue to be the leading platform for gene therapy because of their low relative immunogenicity, broad tissue tropism, and ability to deliver a diversity of therapeutic gene products. Unfortunately, AAV manufacturing faces several challenges with contaminants, drawing increased attention and concern. Following production, AAV preparations undergo a series of diagnostic tests to assess titer, purity, and the presence of DNA contaminants. These DNA contaminants most commonly originate from components used in the AAV production process and encompass plasmid DNA, non-unit length vector genomes, host-cell DNA, and adventitious virus genomes. One type of contaminant that remains poorly understood is replication competent (rc)AAVs. rcAAVs form through genetic recombination events that yield intact, replicative, and potentially infectious virus-like virions. They are detected by passaging clarified lysates from cells transduced by AAV vectors in the presence of helper virus, such as adenovirus. Cellular lysates from the last passage undergo qPCR analyses to quantify rep-containing genomes. This methodology is effective in detecting rcAAVs, but it cannot determine the diversity of recombination forms, nor provide insights into how they originate. We have used AAV genome population sequencing (AAV-GPseq), a method that is based on single molecule, real-time (SMRT) sequencing, to detect and analyze rcAAVs from a test vector designed to treat respiratory disease. Our findings present evidence that sequence-independent recombination between the ITR-containing transgene cassette and the rep/cap plasmid can form from several events. This finding suggests that random recombination during production can form rcAAVs as long as all components for replication and packaging are present. Additionally, we have revised previously held requirements that permit rcAAV formation, which will aid in the development of novel AAV production strategies to further reduce rcAAV contaminants. Importantly, we have developed an advanced and robust method based on next-generation sequencing that can supplement standard quality control measures to ensure the safety of clinical vectors.

778 A Novel Solution Enabling Consistent Quantification of Viral Genome Titer without DNase Treatment

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Adeno-associated viral (AAV) vectors show great promise as a delivery system for gene therapy. However, development of robust methods for quantitation, infectivity and dose-determination has remained a challenge. For quantification of viral genome titer, samples are typically pretreated with DNase to remove vector and packaging plasmid DNAs, and often time followed by proteinase K treatment to remove DNase and release DNase -resistant packaged viral genomes. The challenges using DNase include the incomplete digestion resulting in residual DNA outside the capsids and the inaccessibility of DNase to nucleic acid from partially ruptured viral capsids due to the large size of DNase. To address these challenges with DNase treatment, we developed TruTiter™, a DNase-independent method for quantification of viral genome. The TruTiter™ reagent is a small molecule consisting of a nucleic acid binding moiety, an alkylating group and the molecule is positively charged allowing exclusion from encapsulated biological entities. The crosslinking renders the nucleic acid non-amplifiable by PCR, allowing consistent quantification of viral genomes using dPCR or qPCR. The chemical design of the TruTiter™ reagent is modular in nature. Chemical screens were performed to select an intercalating group as a nucleic acid binding moiety, followed by optimization of alkylating group crosslinking warhead and the charge. After selection

of the lead compound, the concentration of use was determined for various AAV particles. The chemical was effectively excluded from AAV2, 8 and 9 at concentrations less than 100uM. 10uM concentration was used for all studies. The workflow involves addition of the TruTiter™ reagent to a sample for 30 minutes. This covalently crosslinks any nucleic acid that is free or from ruptured virus. Then a neutralization solution is added that stops the crosslinking process, followed by proteinase K treatment to release the protected DNA. The sample is then analyzed using qPCR or dPCR. We assessed the correlation of genome titers determined using the TruTiter™ procedure against the DNAse treatment method and compared the titer values to infectivity. We measured infectivity using a AAV2- NanoLuc® luciferase reporter assay. We generated a standard curve of viral concentrations. By comparing the luminescent value of cells treated with chemical or thermal challenged AAV to the luminescence of the standard curve the viral titer was extrapolated. Tm for AAV2 was 60°C for all the three assays. We then determined the variability in genome quantification measured using the Dnase and TruTiter™ methods. AAV2 were either incubated at 37°C or 65°C for 10 minutes and genome titers determined. For samples treated at 37°C both DNase and TruTiter™ indicated the same value with similar standard deviation. However, for samples treated at 65°C, the DNase treatment showed a two times higher average genome unit measurement and a 2.7 times greater standard deviation, indicative of TruTiter™ method being more robust for genome titer measurements. Next, we treated AAV2 samples with different cell lysis agents typically used in AAV manufacturing process, 0.1% Pluronic, 0.1% Tween and determined genome titers using both DNase and TruTiter™ methods. For both detergents genome tittering showed a greater standard deviation compared to the TruTiter™ method. This study demonstrates the inherent variability in viral genome quantitation when using DNase, and the variability is reduced when using a small-molecule based approach.

779 Early Detection of Tonic Signaling in Primary Human CAR-T Cells by Flow Cytometry

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Chimeric Antigen Receptors (CARs) are intended to trigger T cell activation in response to binding to specific antigens. However, certain CAR designs and excessive levels of CAR expression have the tendency to constitutively activate T cells in the absence of ligands, which has been described as tonic signaling. This chronic spontaneous stimulation has been shown to accelerate T cell differentiation and exhaustion, ultimately leading to dysfunctional CAR-T cells with poor persistence and potency. Therefore, it is highly recommended to screen new CAR constructs for tonic signaling during pre-clinical development. Conventional methods to probe for tonic signaling include detection of CD3ζ phosphorylation in the absence of ligands (e.g. by Western Blot), as well as observation of abnormal T cell expansion kinetics and phenotypes which may manifest after extended culture. However, with the ongoing trend towards shorter processes for CAR-T cell production, earlier, simpler, and less ambiguous readouts are desirable. Here, we propose assessment of CAR ligand-independent surface expression of CD137 as an indicator of tonic signaling. On T cells, CD137 (also known as 4-1BB and TNFRSF9) is a co-stimulatory receptor of the tumor necrosis factor (TNF) receptor superfamily, and its expression strictly correlates with T cell receptor signaling. This property has previously been utilized to identify and isolate antigen-specifically activated T cells in various clinical settings, such as virus-specific T cells in ongoing infectious diseases, tumor-reactive T cells from tumor infiltrates, and alloreactive T cells in transplantation. We show by flow cytometry analysis that this expression pattern also applies to primary human CAR-T cells with tonic signaling through their CAR's CD3ζ endodomain. 72 hours after removal of polyclonal stimulants (e.g. anti-CD3 / anti-CD28 antibody-coated beads), CD137 surface expression remains elevated in T cells transduced with chronically activating CARs compared to untransduced T cells and CAR-T cells without tonic signaling, long before differences in other markers of T cell activation, exhaustion and differentiation (e.g. CD25, CD69, PD-1 and CCR7) become detectable. The kinetics and specificity of CD137 expression, coupled with the simplicity of including it in standard immunophenotyping panels for flow cytometry, make it a useful and attractive tool for pre-clinical characterization of CARs.

780 Development of a ddPCR Protocol for the Quantification of AAV Viral Genomes in Purified Samples

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AAV (adeno-associated virus) is a non-pathogenic virus that is used as a delivery vehicle to transfer therapeutic genes into a patient's cells. Accurate quantification of AAV genome copies is essential for the optimization of the production and purification processes, preclinical studies, and clinical dosage of AAV-based gene therapy products. Several methods exist for the determination of the viral genome (vg) titer with quantitative PCR (qPCR) and droplet digital (dd)PCR being the two most widely used and accepted ones. ddPCR is preferred over qPCR because of the independence of the amplification efficacy, higher precision, and robustness. The aim of this study was to develop a ddPCR protocol for the quantification of AAV viral genomes in purified samples. Unpackaged contaminant DNA, amongst others DNA from the production plasmids and/or cell line, needs to be eliminated before viral genomes can accurately be quantified. We tested several protocols for their ability to remove unpackaged DNA while maintaining accurate packaged viral genome titers. To do so, we spiked plasmid DNA into purified AAV2 vector and evaluated the following sample treatment conditions: (A) incubation with DNAseI only; (B) DNAseI incubation followed by EDTA inactivation, 72°C heat inactivation, proteinase K treatment, and finally 95°C heat inactivation; (C) the same as condition B but with a final heat inactivation at 72°C instead of 95°C (D) no sample pretreatment. Plasmid and AAV genome copies were quantified through a duplex ddPCR (QX200 droplet digital PCR system, Bio-Rad). In contrast to condition D, where no sample treatment was performed, no plasmid DNA was detected in conditions A, B and C. A trend was observed towards lower AAV2 vg titers in condition B compared to conditions A and C (Figure 1). Interestingly, comparable vg titers were noted between conditions A and C. A similar observation was made when repeating the experiment with purified AAV9.

Our findings indicate that heat inactivation at 95°C is detrimental to AAV vg titration and DNAseI treatment only is sufficient for the removal of unpackaged DNA without affecting vg titers. In a next step, we aim to validate this ddPCR protocol and evaluate robustness, specificity, linearity, range, accuracy, precision, and quantification limits.

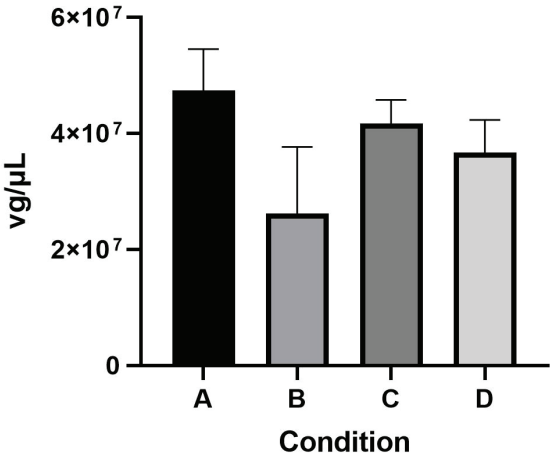

Figure 1. AAV2 viral genome titers after the following sample treatment conditions: (A) incubation with DNAseI only; (B) DNAseI incubation followed by EDTA inactivation, 72°C heat inactivation, proteinase K treatment, and finally 95°C heat inactivation; (C) the same as condition B but with a final heat inactivation at 72°C instead of 95°C (D) no sample pretreatment. N = 8

781 Adeno-Associated Viral (AAV) Vector-Mediated GLB1 Gene Transfer Reduces Substrate Accumulations in a Murine Model of GM1 Gangliosidosis

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Introduction: GM1-gangliosidosis (GM1) is a lysosomal storage disorder resulting from mutations in the galactosidase beta 1 gene (*GLB1*) leading to reduced β-galactosidase (β-gal) activity, neuronal degeneration, and developmental delays. Deficiency of β-gal gives rise to toxic accumulation of its substrates, GM1-gangliosides and other glycoconjugates with β-linked galactose including glycosaminoglycans (GAG, e.g., keratan sulfate) and O- and N-linked glycans (dp5 and A2G2'). Several of these β-gal substrates have been reported to be elevated in GM1 patients and in GLB1-/- mice. However, it is unclear how these substrates would respond to increases in β-gal activity. In this study, we examined the effect of restoration of β-gal activity through AAV-mediated gene transfer in GLB1-/- mice. **Methods and Results:** β-gal activity was measured with a fluorometric assay using a 4-methylumbelliferyl-β-D-galactopyranoside substrate. GM1-gangliosides, an endogenous GAG non-reducing end tetrasaccharide (from keratan sulfate), and glycans dp5 and A2G2' were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). β-gal activity in CSF and serum samples from GLB1-/- knockout mice were <10% of those from the wild-type mouse samples. Conversely, concentrations of GM1-gangliosides in CSF and whole blood, the keratan sulfate tetrasaccharide, and O- and N-linked glycans (dp5 and A2G2') in whole blood and urine were elevated significantly in GLB1-/- mice compared to those in wild type mice. Further, we observed that a single intracerebroventricular injection of an AAV vector expressing human β-gal leads to reduction of GM1-gangliosides in CSF and whole blood and the keratan sulfate tetrasaccharide, dp5, and A2G2' in whole blood and urine. **Conclusion:** These findings demonstrate the potential therapeutic value of the AAV mediated-gene transfer approach and support the use of GM1-gangliosides, keratan sulfate tetrasaccharide, and glycans dp5 and A2G2' as pharmacodynamic biomarkers in clinical studies.

782 Early Development of *In Vitro* Potency Assays for Rare and Ultrarare Disease Gene Therapy Products

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Gene therapies offer efficient treatments for variety of genetic diseases. Potency, as one of the critical quality attributes (CQAs), is a requirement for the successful release of a gene therapy product. Developing an in vitro potency assay can be a challenging and time-consuming process. In this study, we developed a potency assay to measure the expression of a therapeutic gene in vitro using a cell-based system. Multiple cell lines were screened to assess effective transducibility and expression of the gene of interest, utilizing a GFP-based imaging screen approach. Our assay demonstrated good sensitivity, repeatability and reproducibility, accuracy with recovery at all levels tested from 25-200% relative potency, and specificity for the intended gene of interest. The in vitro potency assay developed in this study is a valuable tool for the early development with the capability to be applied in the evaluation of other gene therapy products with minimal program-specific adjustments.

783 Development of a High-Throughput CUT&RUN Platform for Epigenomic Mapping of Rare Primary Immune Cells

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Understanding immune cell differentiation is central to gene and cell therapy research. Regulation of chromatin structure drives many of these processes, leading to the widespread application of chromatin accessibility (ATAC-seq) and transcriptional (RNA-seq) profiling for immune cell characterization. However, these assays often fail to provide mechanistic insight and can lack the granular detail required to define rare and/or novel immune cells (e.g. exhausted T cells). Epigenomic features - such as histone post-translational modifications (PTMs) and chromatin-associated proteins - mark distinct genomic compartments (i.e. promoters) and regulate chromatin structure, gene expression, and cell function. Mapping these features provides a rich context to study cell fate and has great potential for discovering

new biomarkers and drug targets. Notably, the traditional chromatin mapping technology (ChIP-seq) is impractical for immune cell studies due to poor sensitivity, high background, and low throughput. Sample processing methods for ChIP also vary and require substantial expertise, complicating its use in tightly controlled, multi-site studies. Here, we present a breakthrough CUT&RUN assay for rapid, ultra-sensitive profiling of FACS-sorted primary immune cells. Our workflow generates reliable profiles from <10,000 cells per reaction, and is supported by a rigorous optimization strategy, high-quality antibodies, and robust spike-in controls. Further, by automating our CUT&RUN protocol, we were able to increase throughput and standardize sample handling. The resulting workflow was applied at-scale in collaboration with ImmGen, a multi-site project building a comprehensive 'omic database of mouse immune cells. We have generated >1,500 epigenomic profiles from >100 different FACS-sorted primary mouse immune cell types, demonstrating the reliability of our process across diverse samples and labs. Our CUT&RUN approach allows researchers to fully leverage the epigenome to define cell identity. This technology has broad applications in cell and gene therapy research, including CAR T cells, Cas9/dCas9 off-target modifications, exhausted T cells, and induced pluripotent stem cells.

784 CD19-Targeted CAR T Cells Product Identity and Purity Flow Cytometric Method Development: An Analytical Quality by Design Focus

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CD19-targeted chimeric antigen receptor (CAR) T cell gene therapy is a novel, living drug type directed at CD19-expressed B cells in blood cancers. To meet regulatory quality standards, they require drug safety, purity, identity, strength, and potency to be demonstrated before the product can be released. We developed a fit-for-purpose, clinical polychromatic flow cytometric method that supports part of the CD19-targeted CAR T cell drug release criteria for product identity and purity. This method can be used to evaluate process-related cellular impurities. Our method applies the analytical quality-by-design (AQbD) concept which is deeply rooted in various International Conference on Harmonisation (ICH) and United States Pharmacopeia (USP) guidelines. For instance, the associated ICH Q8 (R1), Q9, Q14, and USP 1220 all suggest a AQbD approach to analytical method development for pharmaceutical drug development. Unlike other biologics, gene therapy drug quality is susceptible to varied levels of cellular compositions and functions of incoming apheresis materials and manufacturing process changes. As a result, analytical methods used in such product release evaluations must be sensitive and robust enough to provide meaningful assessments for the process intermediates and product of gene therapies. By the systemic AQbD approach, we followed a series of steps in our method development which can include: the establishment of analytical target profile (ATP); technology selection; identification of critical method attributes (CMAs) through a risk assessment; method development and optimization; method operable design region (MODR); and method qualification. Through this approach, we identified and verified critical quality attributes (CQAs) that can affect the drug potency. In addition, we developed and qualified this method using frozen CAR-T cell drug product lots and cell lines on a BD FACS Lyric™ cytometer to accommodate a high demand for analytical testing. Finally, our results demonstrate that the developed method is reproducible, sensitive, robust, and compliant, appropriate for product lot release testing. We will continue to collect more data to establish drug product specifications for the CQAs.

785 Predictive Assays for Modeling Foreign Body Response to Encapsulated Cell Therapies

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The Foreign Body Response (FBR) to encapsulated cell therapies poses a great challenge to developing a long term durable therapeutic product. The severity of this response is attributable to: a) the nature of the biomaterial; b) the secretome profile of the cell and; c) the immunogenicity of the protein cargo of the encapsulated cells. Sigilon's Shielded Living Therapeutic™ (SLTx) technology uses Afibromer™ conjugated alginate spheres that are designed to be biocompatible and to avoid FBR thus allowing longer therapeutic durability. We developed a set of *in vitro* and *in vivo* assays designed to guide us in selecting more durable configurations for our therapeutic programs. Macrophage attachment, the initiating step in the FBR process, is influenced by the nature of the biomaterial and the inflammatory secretome of encapsulated cells. Using a sequence of secretome analysis, coupled with a high-throughput *in vitro* macrophage attachment assay, we are able to faithfully model this process and de-risk our product *in vitro*. Then, using a humanized mouse model that faithfully recapitulates the FBR process we can predict the FBR potential of our encapsulated cell therapy. These predictive assays guide us in the development of a durable allogeneic cell therapy product.

786 Eliminating Challenges in Lentiviral Vector Characterization with Capillary Electrophoresis

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Lentiviral vectors (LVV) have become a prominent and popular gene delivery system for both in vivo and ex vivo therapies. It is crucial to identify, characterize, and quantify these LVV particles to ensure quality, safety, efficacy, and to meet all regulatory requirements. In this work, we leveraged the Maurice CE-SDS platform to develop a robust method for LVV analysis. We show the method can be used for LV identity and viral titer. Comparing different LV vendors, unique but similar profiles were obtained with the CE-SDS method. Assessing and characterizing the capsid core protein p24 offers an estimation of

LVV quantitation as well as transduction efficiency. Using recombinant p24, we show accurate viral titer estimation. Together, the LV method provides a rapid and accurate analysis of lentiviral products.

787 Development and Comparative Assessment of a Rapid, High-Throughput Method for Accurate Quantification of Host Cell DNA Impurities in Final Drug Product and Process Intermediates

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Biologics manufactured in cell lines carry inherent risk of incorporating host cell material in the drug product. In particular, elevated levels of residual host cell DNA (hcDNA) contaminants increases oncogenic risk and may lead to a systemic induction of the immune response. The World Health Organization and European Pharmacopoeia have established a guideline for the acceptable level of hcDNA (<10 ng per dose) in biologics. As such, regulatory agencies have focused on quantifying and characterizing hcDNA in gene therapy products as a critical parameter for product safety. Therefore, a highly sensitive, accurate, and robust method of hcDNA quantitation provides valuable information during process development and is essential as a safety and quality metric of the final drug product. In this work, we report a robust, high throughput qPCR method capable of accurately quantifying total and encapsidated hcDNA in all process intermediates and final drug products within two hours. When compared to a validated, commercial residual hcDNA qPCR kit, the assay exhibits improved accuracy (>90%), specificity/sensitivity (6 log dynamic range, LLOQ-30 fg), and reproducibility (<20% inter-assay RSD) in several unrefined in-process matrices. We have shown that our developed residual hcDNA qPCR assay is suitable to reliably quantitate low levels of host cell impurities without extensive sample processing for intermediate and final drug product samples.

788 Evaluation of a Synthetic Regulatory T Cell Platform Using a Suite of Analytical Methods and Preclinical Models

Ashley Mahne, Jesus Banuelos, Andrew Tremain, Shriya Sethuraman, Shairaz Shah, Candice Gibson, Simone Sandoval, Jazmin Bravo, Kelsey Phipps, Baden Dense, Jeanne Flandez, Jennifer Zeng, Dominic C. Borie, Jeffrey Greve, John Lee, Peter Beemiller, Charles Kaplan, Brandon Kwong
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Introduction: Regulatory T cell (Treg) therapy is a promising avenue for the treatment of autoimmune diseases. Tregs exert potent, polyfunctional immune suppression and have the potential to reset immune tolerance in a variety of inflamed tissues. Two major challenges in translating Treg cell therapies into the clinic have been their polyclonal repertoire and the limited number of purified, stable Tregs that can be isolated from disease patients, expanded, and manipulated ex vivo. Synthetic regulatory T cells (synRegs) derived from abundant $CD4^+$ T cells are an attractive alternative with more facile manufacturability. To ensure the safety and efficacy of any Treg or synReg therapy in the clinic, deep characterization of its functionality and stability is essential. Here, we report on the analytical characterization of a novel chimeric antigen receptor synReg platform (CAR-synRegs). **Methods**: CAR-synRegs or synRegs were generated by transducing human naïve $CD4^+$ T cells with FOXP3, along with or without a 2nd generation CAR targeting a tissue antigen, encoded in a single lentiviral vector. Transduced cells were expanded in vitro to attain clinically relevant doses using a GMP-adaptable process. In vivo stability and suppressive efficacy of polyclonal human synRegs were evaluated in a xeno-GvHD mouse model. To further mimic the inflammatory signaling environment likely to be experienced by CAR-synRegs in a disease patient, CAR-synRegs were exposed to homeostatic or pro-inflammatory conditions in extended cultures in vitro, and restimulated in a polyclonal or CAR-specific manner. Following culture under such conditions, CAR-synRegs were evaluated for their ability to suppress effector T cell responses and dendritic cell function, maintain stable CAR and FOXP3 expression, and secrete pro- or anti-inflammatory cytokines. Moreover, synRegs were profiled for both Treg-associated gene and protein expression by RNAseq, DNA methylation analysis, and flow cytometry. **Results:** Deep analytical characterization demonstrated that CAR-synRegs express both a Treg-like profile and functional suppressive activity. CAR-synRegs maintained high FOXP3 expression and suppressed effector T cell responses and DC functions in vitro, while polyclonal synRegs potently inhibited disease progression in vivo in the xeno-GvHD model. However following exposure of CAR-synRegs to inflammatory factors, CAR engagement induced the increased production of Th1 effector-associated cytokines, indicating an opportunity to further reprogram CAR-synRegs for maximal safety and efficacy. Thus CAR-synRegs were re-engineered with the modular addition of genetic and non-genetic (small molecule) modifiers, and evaluated using the same suite of analytical methods to identify modifiers that improved both the functional and safety profiles of CAR-synRegs. These modifiers increased long-term FOXP3 stability, upregulated the expression of Treg-associated functional receptors, and/or modulated the pro- vs anti-inflammatory cytokines secreted in response to CAR activation and inflammatory conditioning. **Conclusions:** The risk of Treg instability is a concern that is applicable to both synthetic or naturally-derived Treg therapies. These results highlight our ability to identify potential limitations of Treg therapies and address such risks via genetic and non-genetic reprogramming approaches to enhance the efficacy and safety profile of a Treg platform.

789 NPlasmid-seq: A Quality Control Toolkit for Full-Length Plasmids Enabled by Nanopore Sequencing

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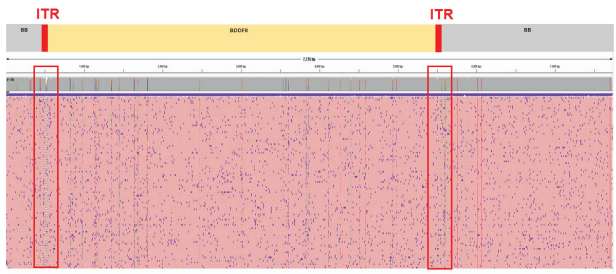
In current gene therapy, the prevailing delivery vectors are engineered viruses produced in packaging cells by transfecting plasmids. Sequence verification and contamination control of plasmids are instrumental to vector quality assurance. The short reads of Sanger sequencing and

Illumina NGS pose a challenge in piecing together the full plasmid sequence, particularly for vectors containing repetitive fragments. Furthermore, these DNA amplification-based sequencing approaches often fail to read through strong secondary structures like AAV ITR. In addition, no documented evidence has shown that the first two-generation methods can confidently identify small contaminants (e.g., 5%). To ensure vector authenticity during plasmid manufacturing and vector packaging, the gene therapy field calls for a protocol to determine the full length and to detect trace amounts (e.g., 0.1%) of contaminated plasmids. Nanopore sequencing (NP-seq) technology reads ultralong sequences at a single molecule level, providing an opportunity to resolve such challenges. **Methods**: Here, we introduce the experimental procedure and bioinformatics pipeline to achieve that goal. To prepare for the NP-seq library, we first linearized plasmids using RNPs of Cas9-sgRNAs targeting the plasmid backbone, followed by sequencing using R9.4.1 flow cells on a MinION. In addition, over ten sgRNAs were designed to enable pooled sequencing of different batches. To streamline data analysis, we developed a python-based pipeline termed NPlasmid-seq, which outputs demultiplexed reads, contaminated plasmids, computed consensus sequences, and vector visualization results in one folder. A stretch of DNA flanking the CRISPR cleavage site was used to demultiplex the pooled reads. **Results**: Plasmid-seq could determine full-length lentiviral vector plasmids over 10 kb, adenoviral plasmids of 30-35 kb, AAV plasmids with the ITR structure, and mRNA in vitro transcription (IVT) template plasmids with consecutive 90 adenine bases (A90). When a reference is not provided, the consensus computed with over 100 reads was around 99.5% identical to the known sequence, while inputting a reference leads to correct alignment with 99.9% of sequences. To determine possible plasmid contamination, NPlasmid-seq first teased out reads with indels of over 10 nt and binned to a "Warning Data" collection, followed by aligning with the reference sequence library. To mimic plasmid contamination, we manually doped with 0.1% of an unknown plasmid. To decrease the false discovery rate, we called contamination only with the support of at least three reads. We found that over five reads of contaminants were sufficient to parse out its identity at 99% confidence. **Conclusion**: NPlasmid-seq, in its current version, cannot replace Sanger sequencing due to the high error rate of NP-seq. However, it provides a powerful toolkit to determine full-length plasmids and minute amounts of potential contaminants. Therefore, NPlasmid-seq offers a full plasmid sequencing and data analysis pipeline that would find its application in vector cores or gene therapy companies.

A representative of AAV plasmid sequence verified by NPlasmid-seq



790 Establishing GMP Compliant Adenovirus Serotype 35 Manufacturing Process

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As gaining global recognition of gene therapies for its remarkable clinical benefits in a variety of medical applications, research and development of viral vectors as medical applications have become considerable interest. Among the different types of viral vectors, adenoviral (Ad) vectors have been extensively studied and used for a long time in medical care, especially recent worldwide attention due to the recent approvals of Ad vector-based COVID-19 vaccines. We are developing a novel adenovirus serotype-35 (Ad-35), to see if it could be useful as a vaccine (non-replicating vector) or oncolytic virus. So far GMP compliant manufacturing process of Ad-35 has not been established in the world.This time, we challenged to establish Ad-35 vector GMP compliant manufacturing process development for the usage in the investigational clinical trial. Based on the pre-conditioning analysis of optimizing culture condition using the suspension HEK293 cell line with serum-free culture medium, a closed mass cell culture system was constructed by transfer to the shaking back-type closed culture system. Final 5,000 mL of suspended HEK293 cells Ad-35 vector-infected were obtained. Virus-infected whole culture cells were lysed to obtain viruses, following further purification and concentration steps were performed to get crude harvest. Subsequently, target fraction separation by ion exchange membrane chromatography was performed to capture Ad-35 vector, resulting in final 50 mL viral-contained products collected. We could identify the critical process parameters in the up-stream (closed mass cell culture system) and down-stream (virus purification process). These clinical developments would suggest an excellent path to construct a GMP-compliant viral investigational drug production platform.

791 Clinical-Scale Production of Lentiviral Vectors at a New Academic cGMP Facility

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Lentiviral vector (LV)-mediated gene delivery has shown clinical promise in the gene and cell therapy field. LVs offer a high rate of transduction into many cell types, the ability to transduce non-dividing cells, stable gene integration, and a reduced risk of insertional mutagenesis when compared to gamma-retroviruses. However, the production and purification of high-quality LVs for clinical trials remains challenging. The MCW/BCW GMP Vector Production Facility's mandate is to produce high-titer, clinical-grade LV for use in early-stage trials at the Milwaukee Regional Medical Center (MRMC). Herein we describe a method to scale-up production of high-titer LVs that are capable of transducing multiple cell types under GMP conditions. We package our LV using HYPER technology, a commercially available, fully closed and disposable system composed of multiple layers of a gas-permeable, tissue culture-treated, growth surface. Following packaging, we purify LV by post-harvest clarification and Mustang Q anion-exchange membrane chromatography. Scale-out experiments have provided encouraging recovery yields from

clarification (83%) and Mustang Q chromatography (67%) resulting in a 1000-fold volume reduction with excellent viral recovery (60-70% of starting input LV). The chromatography eluate is subsequently diluted and concentrated using tangential flow filtration (TFF) and treated with Benzonase endonuclease (50 U/ml) at 37C. TFF is then repeated to further concentrate the LV down to 200 ml of deliverable product. To evaluate compliance with current FDA guidance for clinical application, we analyzed samples from our production and purification process to assess LV purity and titer. We report functional titers between 1.3 x 10^7 TU/mL and 2.2 x 10^8 TU/mL for clinically relevant vectors. Our purification method results in minimal endotoxin detected (<100 EU/ml). Furthermore, post purification residual plasmid DNA, host-cell DNA, and host-cell protein were reduced >98%, >99%, and >98%, respectively. The HYPER LV packaging strategy, Mustang Q chromatography, and TFF purification regimen described here is a practical method for LV scale-up to early-stage clinical trial volumes using affordable disposable laboratory supplies.

792 Cell Line Developement for High-Titer Lentiviral Vector Production

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Lentiviral vector (LV) is an important tool in gene delivery and gene therapy. Current production of lentiviral vectors is based on multiple plasmids co-transfection of adherent HEK293T cells in monolayer flasks. However, the cell population is heterogeneous and not achieving ideal yield. It is possible to improve the packaging capacity of production cells to increase the yield of lentiviral vectors per cell. In this study, we report a method to produce LVs in high yield by developing monoclonal HEK293T cell line. This method can sort out single HEK293T cell with high transfection efficiency and seed the cell into one well of a plate. The system uses low fluidics pressure, resulting in a higher frequency of wells containing viable and single cell with high transfection efficiency. Ten cell lines developed from this system were finally selected. Using the selected HEK293T cell lines, LVs production was tested in 10-layer cell factories and the vector titer was quantified. Four cell lines demonstrated a double yield comparing with the original cell line. One cell line was further tested by packaging different GOI, there was 2-4 folds improvement in yield across different GOI packaging.

793 High-Performance, Scalable Lentiviral Vector Manufacturing Workflows Supporting Continuous Processing

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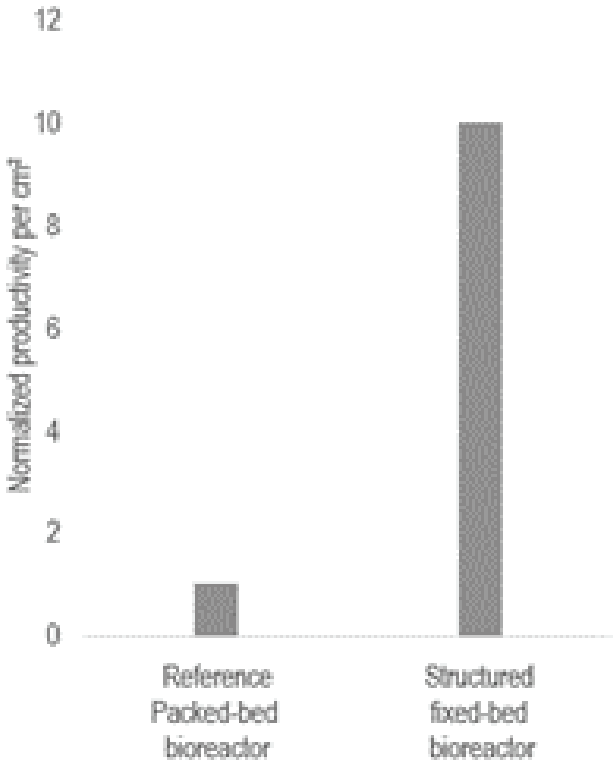
Due to the clinical successes associated with gene-modified cell therapies such as CAR-T, and given rising interest for in vivo gene therapy applications, the demand for lentiviral vectors (LV) is expected to continue growing significantly in the coming years. LV manufacturing however presents unique challenges, due to the highly sensitive nature of the product and the inherent complexity of its manufacturing process. In addition, as required production throughput can vary significantly with dose size, target indication and development stage, technologies providing flexibility on scale while maintaining reproducibility are a key requirement for developers and manufacturers. While much effort has been invested in developing manufacturing workflows based on suspension processes in stirred-tank reactors (STR), this approach remains associated with several limitations. Indeed, key parameters such as shear, mixing and gas exchange cannot be simultaneously maintained across scales, constraining developers and impacting the reproducibility and cost effectiveness of manufacturing processes. In this session, the speaker will present a streamlined platform designed to enable flexible, scalable LV manufacturing. Several cases studies will be highlighted, demonstrating how structured fixed-bed technology can address specific challenges associated with LV production in both adherent and suspension platforms. The speaker will illustrate how a low shear, homogenous bioreactor environment leads to a >10-fold increase in productivity, and present data supporting maintained performance across scales. Application of perfusion processes and continuous processing approaches will be explored, laying out a scalable, cost-effective workflow for clinical and commercial stage LV manufacturing. Finally, the speaker will touch on considerations regarding intensified seed train generation for large-scale production, with the presentation of automated, closed system addressing key challenges associated with this critical step.Figure 1. Increased LV functional titre in a structured fixed-bed bioreactor. Lentivirus production in reference (packed-bed bioreactor process) compared to a structured fixed-bed bioreactor process/ Normalized functional titre (TU) is displayed, relative to the reference process



794 LentiVEX Packaging Cell Line v2.0 Yields Comparable LVV Titres to the 4-plasmid Process

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Current lentiviral vector (LVV) manufacturing approaches primarily use fully transient and adherent processes which present challenges of robustness, scalability, and high cost-of-goods. To address these, we have developed suspension cell lines for transient, semi-stable and stable LVV manufacture. Tet-regulated VSV-G and Gag-Pol, and constitutive but codon optimised Rev lentivirus packaging components were randomly integrated into WXATUS0028 suspension HEK293 cell line. Using a fully traceable, animal component-free and high-throughput cell line development process, the top LentiVEX Packaging cell line v2.0 was identified. It only requires transfection of the LV genome plasmid with doxycycline induction to achieve LVV yields of 2 x 10^8 TU/mL (with eGFP as Gene of Interest), comparable to the four-plasmid process with WXATUS0028. Long term production stability of the packaging cell line was assessed and LVV titres were consistent (> 1.5 x 10^8 TU/mL) for 27 passages without any selective pressure applied. Genetic stability and location of the integrated genes are currently being assessed by ddPCR, NGS and TLA analysis. Therapeutically relevant GOIs of sizes ranging from 0.05 to 4.4 kb were also tested, and the packaging cell line demonstrated higher yields than the WXATUS0028 baseline when GOI > 1.7 kb. Scale-up vector production and process development with the packaging cell line are ongoing, with the Master Cell Bank (MCB) being generated for GMP-standard vector manufacturing. With a reduced plasmid cost and a simplified transfection process, the LentiVEX Packaging Cell Line v2.0 is a flexible platform to support both early-phase discovery studies with easy screening of multiple GOIs and large-scale LVV manufacture.

795 ADEVO: Proof-of-Concept of Adenovirus Directed EVOlution by Random Peptide Display on the Fiber Knob

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Introduction: Directed evolution is a method of vector development consisting in the generation of a random library of vector variants, followed by the selection of improved variants according to pre-defined selection criteria. Directed evolution already led to numerous innovations in Adeno-associated virus or Lentivirus vectors but yielded few successes in Adenovirus vector (AdV) development until now. This was mainly due to technical difficulties in constructing large random libraries. Meanwhile, AdVs clinical applications as gene therapy or oncolytic vectors are still hampered by the predetermined tropism of natural serotypes. To overcome this challenge, we hypothesized that validated vector retargeting technologies such as peptide insertions can be incorporated into the AdV toolbox. Here we designed novel Adenovirus Directed EVOlution (ADEVO) protocols for AdV retargeting, based on the insertion of random peptides in the fiber knob domain. To assess and optimize the protocols, we constructed libraries based on the frequently used adenovirus type 5 (Ad5) and tested if we could select variants able to infect A549-ΔCAR cells, that are non-permissive for natural Ad5. Methods: In protocol 1, random oligonucleotides were inserted through NEBuilder homologous recombination inside a plasmid containing the AdV fiber gene, which was then transferred to a full AdV genome. In protocol 2, the genome library was built in a single step through direct insertion of random oligonucleotides into the AdV genome. In both protocols, the initial vector library was built by transfection of the genome library into HEK293 cells. Variants with improved tropism for A549-ΔCAR cells were then selected by serial passage of the libraries on these cells. Library complexities were estimated by NGS. Results: Cross-packaging is a process by which capsids do not package their corresponding genome. This can lead to selection failure in directed evolution. Using co-transfections of wild-type and fiber-deficient adenovirus genomes, we determined that the cross-packaging rate of adenoviruses remained below 3% when applying up to 5000 transfected genomes per cell, a dose at which high vector titers can be produced. Therefore, cross-packaging should not hamper library construction and selection.ADEVO protocols 1 and 2 enabled the construction of high complexity genome and vector libraries, with at least **2.7e5 and 3.9e5 unique variants** in the respective initial vector libraries. Vectors able to efficiently infect A549-ΔCAR cells were enriched during the selection phase, while non-functional variants containing stop codons were eliminated. In subsequent steps, enriched variants will be purified and characterized in detail. Conclusions: ADEVO, our novel directed evolution workflow for Adenoviruses, facilitates the construction of highly diverse variant libraries and the selection of improved vectors with user-friendly protocols. We expect that this new technology can contribute to the development of clinically relevant adenoviral vectors with more specific tropism and higher transduction ability in target cells.

796 Vector Manufacturing Processes for Gene Therapy

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AGC biologics is a leading global CDMO, providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells. Adeno Associate Vectors (AAV) and Lentiviral vectors (LVV), produced with transient transfection in HEK293 and HEK293T cells are extensively used for genetic modification in gene therapy for *ex-vivo* and *in-vivo* treatments. An important manufacturing challenge is the availability of a efficient and scalable off-the-shelf vector production processes. To address this goal AGC Biologics is developing different systems for AAV and LVV production at large scale, in adhesion and suspension, flexible to be adapted to different GOI and AAV serotypes. Adherent platforms exploit Pall iCELLis® fixed-bed disposable bioreactors. Suspension platforms are based on proprietary cell lines in a culture system without animal derived reagents in stirred tank bioreactors. AAV manufacturing process in adhesion has been scaled up on 333m^2 bioreactors with the production of both retained and released serotypes starting from

70L and 480L of bulk lysate respectively. We obtained a productivity at full scale of 1.6E+09 VG/cm^2 for a retained serotype and 1.2E+10 VG/cm^2 for a released serotype . DSP includes concentration step by TFF, affinity chromatography, polishing by AEX to enrich in full/empty particles, further concentration and diafiltration steps and final sterilizing filtration. Total DSP yield ranges between 30% and 40%. AAV production in suspension has been performed at 50L pilot scale in Sartorius Biostat STR bioreactor obtaining 1.1E+11 VG/mL with 70% ratio full/total and a yield ranging between 20% and 30%. Scale up to 200L will be performed in 3Q 2023 and the process is ready to be scaled up to 2000L. AGC Biologics (former MolMed) has a long lasting experience in the manufacturing of LVV for clinical and commercial phases. As evolution of the well-established adherent GMP 48L CF process we developed a robust, scalable, high quality and quantity processes for the industrial scale production of LV vectors performed in iCellis500 bioreactor 133m^2 obtaining 200L of bulk vector with an average titer of 3x10^7 TU/mL. DSP foresees DEAE chromatography, concentration and diafiltration by HF and final sterilizing filtration with an overall yield of 20-30%. LVV manufacturing suspension process has been developed at scale down and confirmed at 50L scale obtaining 5.7x10^7 TU/mL in the bulk vector. Downstream process was designed in order to accommodate larger volumes of vectors from bioreactor. Single use Tangential flow filtration and DEAE chromatography steps were successfully implemented, with good removal of process related contaminants and no detrimental impact on vector infectivity and stability. Freshly released affinity resins for LVV are under evaluation in order to increase purity and quality of final vector for in-vivo application. AAVs and LVVs have been characterized with an orthogonal strategy including Viral Genome/Particles quantification, infectious viral titer, core protein characterization and process related impurities demonstrating high potency and quality suitable for ex-vivo and in-vivo applications. Moreover, the possibility to leverage the analytical platform already in use for the GMP process accelerates transition from development to GMP clinical and commercial productions.

797 A Scalable, Suspension-Based Platform to Produce Virus-Like Particles (VLPs) for *In Vivo* Gene Therapy

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Helper dependent adenovirus (HDAd) is a virus-like particle (VLP) capable of safely delivering large payloads *in vivo*. HDAd vectors are completely devoid of viral coding sequences, thus require a helper virus and an engineered HEK293 cell line to replicate. The established production process utilized an adherent cell line grown in serum-containing medium, which is more challenging to develop and scale compared to suspension processes. The engineered HEK293 cell line was weaned off serum and adapted to grow in suspension culture. Exponential growth and successful HDAd production were demonstrated in shake flasks. Ambr15 experiments were conducted to screen and optimize critical process variables for HDAd production. Shake flask and Ambr15 data demonstrated comparable per cell HDAd production in suspension culture compared to that of the original cell line. Using Ambr15 data, the suspension process was scaled to 2L benchtop bioreactors. Bioreactor HDAd production was comparable with Ambr15 data, demonstrating process scalability.

798 Trial in Progress: A Phase 1 Trial of TSC-100 and TSC-101, Engineered T Cell Therapies That Target Minor Histocompatibility Antigens to Eliminate Residual Disease After Hematopoietic Cell Transplantation

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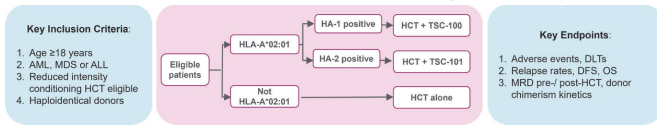
[1]City of Hope, Duarte, CA,[2]Hackensack Medical Center, Hackensack, NJ,[3]Mount Sinai, New York, NY,[4]Karmanos Cancer Institute, Detroit, MA,[5]TScan Therapeutics, Waltham, MA,[6]Columbia University, New York, NY

Engineered T cell therapies such as chimeric antigen receptor T (CAR-T) cell therapies have transformed the treatment of lymphoid malignancies but have not yet proven effective in myeloid malignancies. Allogeneic hematopoietic cell transplantation (HCT) remains the best curative option for these malignancies but ~40% of patients relapse post-HCT with ~90% mortality due to residual disease post-HCT. A key challenge in these malignancies is identifying the right antigens for T cell targeting. Cancer-associated antigens have heterogenous expression enabling rapid escape of malignant cells with low antigen expression while targeting lineage-specific antigens without a clear distinction between malignant from normal myeloid cells carries the risk of prolonged neutropenia. A potential solution is to target minor histocompatibility antigens (MiHAs) that are highly and homogenously expressed on all hematopoietic cells and are mismatched between patients undergoing HCT and their donors. These mismatches enable T cells to selectively eliminate residual patient hematopoietic cells, normal or malignant, leaving donor cells untouched. TScan has developed allogeneic donor derived T-cell products TSC-100 and TSC-101, targeting MiHAs HA-1 and HA-2 respectively, both presented on HLA-A*02:01. By choosing HCT patients who are HLA-A*02:01 positive (>98% of whom are either HA-1 or HA-2 positive) and donors who are either HLA-A*02:01 or MiHA negative, TSC-100 or TSC-101 can eliminate residual patient-derived hematopoietic cells after HCT to prevent disease relapse. Study NCT05473910 is a multi-center, multi-arm, non-randomized controlled Phase 1 umbrella study evaluating the feasibility, safety and preliminary efficacy of TSC-100 and TSC-101. Inclusion criteria include adults with AML, MDS or ALL eligible for reduced intensity conditioning-based haploidentical donor transplantation from HLA or MiHA mismatched donors. HLA-A*02:01-positive patients undergo HA-1/ HA-2 testing and are assigned to either TSC-100 or TSC-101 treatment arms in addition to standard HCT. HLA-A*02:01-negative patients in the control arm receive standard HCT alone. Upon count recovery after HCT, patients in the treatment arms receive either TSC-100 or TSC-101, administered as a single or two doses. Primary endpoints include adverse event profiles and dose limiting toxicities. Secondary end points include relapse rates, disease-free survival and overall survival. Exploratory endpoints include surrogates of efficacy

such as minimal residual disease (MRD) rates and donor chimerism rates and kinetics. MRD is measured before HCT, after HCT and after T cell therapy using a combination of flow cytometry and next-generation sequencing (NGS) that detect MRD with greater sensitivity than either assay alone. Donor chimerism is measured by standard STR-based and novel high-sensitivity NGS-based assays to quantify residual patient-derived hematopoietic cells. Together these assays measure elimination of residual patient hematopoietic cells, malignant or normal, and provide early evidence of biological activity.



799 Navigating the EU Clinical Trial Regulation (536/2014): Key Considerations for Gene Therapy Medicinal Product (GTMP) Developers

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As of January 31st, 2023, all new EU clinical trial applications (CTAs) must be submitted under the EU Clinical Trial Regulation (CTR) (535/2014) and via the Clinical Trials Information System (CTIS) portal. The regulation was created to prioritize the rights, safety, dignity, and well-being of clinical trial participants while simplifying and harmonizing the submission of CTAs and conduct of clinical trials in the EU. In the first 11 months of CTR implementation, only 4 clinical trials evaluating gene therapy medicinal products (GTMPs) have a decision out of 214 total clinical studies with a decision (EMA/2/2023, 31 January 2023, edition 9). It is expected that submissions for GTMPs and all clinical studies will accelerate in the next year with the requirement to submit initial CTAs under the CTR. Given the lack of precedent for GTMP developers, we have identified several key issues to consider when strategizing an EU clinical trials approach under CTR. Issues directly related to the CTR include member state concerned selection strategy, the management of auxiliary medicinal products, navigation of transparency requirements, and management of review and modification procedures. Other issues impacting CTR strategy include timing and expectations for EU Qualified Person interactions, genetically modified organism application considerations, and in vitro diagnostics considerations. In this presentation, Forge Biologics (Forge) will discuss potential solutions for GTMP developers to navigate these key issues. Forge continues to progress its own therapeutics programs in the EU and will use this experience to support our clients in the navigation of the CTR environment.

800 A Rollercoaster of High's and Lows; Learning from One Patient's Journey to CAR-T

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The diagnostic and treatment journeys leading up to consideration of cell and gene therapies, such as CAR-T, are significantly varied from person to person. When it comes to clinical trials, cell and gene therapies are typically studied in patients who have already had a long diagnostic and treatment journey and are at the point where they have exhausted all other care options. So what can we learn from one patient's journey? The team conducted a patient insights project to provide a deeper understanding of awareness, motivations, hesitations, and general experiences regarding a patient's journey before, during and after gene/CAR-T therapy for their cancer that can guide our strategies related to cell and gene therapy clinical trials. During this project, we performed an in-depth interview with representative patients who had received CAR-T cell therapy. The patient was diagnosed with supraclavicular lymph node stage 2 in 2011 and initially underwent 6 cycles of R-CHOP chemotherapy. In 2015, the patient was diagnosed with extra nodal Waldeyer's Ring and underwent further treatments with R-ICE, intrathecal methotrexate and head and neck radiation. Treatment options had limited success and the patient developed a number of severe side effects from their disease and treatments, including sepsis, severe dysphagia with mouth ulcers leading to significant weight loss. The patient's final treatment option per standard of care was allogenic transplant, however, no match was found. The patient became aware of CAR-T cell therapy clinical trials through a patient community, and as a last resort sought to enroll in a trial. Eventually, the patient was only accepted onto a trial in 2016 as a participant had died. As a study participant, the patient shares with the Patient Insights team what it was like to navigate a barrage of complex medical information, and make decisions with implications far beyond what is comprehendible for people who haven't been patients. As a last resort option, the journey through CAR T therapy was physically and emotionally exhausting - but found herself at the end, in remission but unsure of what her future would look like. What can we learn from the patient journey that may better the way we conduct clinical research? By shifting our focus from product led, to patient led drug development, we can help enhance the patient experience and ultimately improve patient outcomes.

801 One-and-Done Gene and Cell Therapy for Familial LCAT Deficiency Using LCAT Gene Transduced Human Adipocytes

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Mutations in the gene encoding lecithin: cholesterol acyltransferase (LCAT) cause familial LCAT deficiency (FLD) characterized by severely lowered HDL-cholesterol, corneal opacities, hemolytic anemia, and renal disease. The prognosis for FLD is unfavorable, and patients finally develop renal failure. Enzyme replacement therapy is not currently available for this disease. The use of proliferative preadipocytes is advantageous in ex vivo gene therapy. After liposuction of fat, subsequent collagenase treatment, and floating centrifugation, preadipocytes can be readily prepared. The resulting preadipocytes have a highly proliferating rate that allows high transduction efficiency of the foreign genes and obtaining many cells for implantation. Once implanted, the cells undergo final differentiation into adipocytes that are highly stable in the body and have high secretion potential. To treat FLD, we performed first-in-human autologous implantation of genetically modified adipocytes (GMAC) expressing LCAT (LCAT-GMAC) into a patient with FLD. This first-in-human autologous implantation of LCAT-GMACs was shown to be safe by evaluating

adverse events. The LCAT-GMAC implantation increased serum LCAT activity by approximately 50% of the baseline and sustained over several years. Consistent with increased LCAT activity, intermediate-density lipoprotein (IDL) and free cholesterol levels of the small and very small HDL fractions decreased. We found the hemoglobin/haptoglobin complex in the hemolyzed pre-implantation sera of the patient. After one week of the implantation, the hemoglobin/haptoglobin complex almost disappeared. Immediately after the implantation, the patient's proteinuria decreased temporarily to mild levels and gradually increased to the baseline. At 48 weeks after implantation, the patient's proteinuria deteriorated with the development of mild hypertension. By the treatment with antihypertensives, the patient's blood pressure normalized. The proteinuria rapidly decreased with the normalization of blood pressure to mild proteinuria levels. The GMAC-LCAT treatment is shown to be safe in this study. It will provide novel therapeutic reagents in future medicine, including treating various inherited diseases and metabolic disorders.

802 Sustained Dystrophin Expression in an Infant with a *DMD* Exon 2 Duplication Following Dosing with scAAV9.U7-ACCA

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Duchenne muscular dystrophy is a life-limiting disorder caused by pathogenic frame-truncating variants in the *DMD* gene. Exon 2 is the most common exon duplication, and complete exclusion of exon 2 results in translational re-initiation via a downstream internal ribosome entry sequence, making it a promising target for an exon skipping therapy. We developed an AAV-mediated exon skipping vector (scAAV9.U7-ACCA) comprising four copies of a modified U7snRNA containing antisense sequences targeting the splice donor (2 copies) and splice acceptor (2 copies) of the *DMD* exon 2. We previously reported results of our open-label first-in-human study in three boys with out-of-frame exon 2 duplications. Here we report the 15-month safety and efficacy data of the youngest participant, who is the first DMD infant to receive an AAV-mediated gene therapy. Following uneventful intravenous delivery of 3.0x10^13 vg/kg at 7 months of age, he has continued to meet all developmental milestones on time and his Bayley-IV motor scores remain in the normal range at 15 months post-transfer. At 12 months post-transfer, serum creatine kinase had dropped by 91% from screening (to 1079 U/L), and muscle biopsy showed highly efficient and persistent exon skipping (in 99% of mRNA transcripts). Dystrophin protein expression was similarly robust and sustained, with immunofluorescence showing ~95% dystrophin positive fibers and western blot showing levels of 88.3% of normal (increased from 70.1% at the month 4 biopsy). We conclude that treatment with scAA9.U7-ACCA in infancy is safe and robustly efficacious with therapeutic effect sustained over at least 12 months, supporting continued clinical investigation.

803 Initial Findings of Retinal Gene Therapy in Patients with Bietti Crystalline Dystrophy

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Background: Bietti crystalline dystrophy (BCD) is an incurable autosomal recessive inherited disease that leads to blindness due to mutations in the CYP4V2 gene, a gene belongs to the functional cytochrome P450 gene family (family 4, subfamily IV, polypeptide 2). Defectiveness in CYP4V2 function causes retinal pigment epithelium cell damage leading to degeneration of the retina. Thus restoration of normal CYP4V2 gene expression with adeno-associated viral (AAV) vectors can be a therapeutic strategy for BCD. We report herein the preliminary results of an ongoing exploratory study evaluating safety and efficacy of the subretinal gene replacement therapy product VGR-R01 by rAAV8 delivery of a functional CYP4V2 transgene. **Methods:** Three participants (aged 34-48 years; 1 female) with BCD resulted from biallelic mutations were sequentially assigned to one of 3 dose levels before receiving a single subretinal injection of VGR-R01 ($0.2\text{-}1.2^{10}$vg, 0.1mL per eye). Prior to VGR-R01 injection, participants received glucocorticoids for immunosuppression to decrease the risk of vector-related immune responses. Adverse events (AEs), visual function assessments including best corrected visual acuity (BCVA), multi-luminance mobility test (MLMT), static visual field testing, and full field electroretinogram, etc. were monitored throughout the study. This study is registered with ClinicalTrials.gov, number NCT05399069. **Results:** The functional vision outcome, measured by MLMT scores of the study eyes, rapidly increased by 2-points in patient 1, 1-point in patient 2, and stable in patient 3 in around two weeks upon injection of VGR-R01. Additionally, the collateral and bilateral MLMT scores changed by 0-2 points. The baseline visual acuity in all the study eyes were in the range of off-chart vision. Two weeks after administration of VGR-R01, in all patients' study eyes and collateral eyes, BCVA changed by 0-0.3 LogMAR (with the scale adapted from Holladay used to assign values for off-chart acuities). Moreover, VGR-R01 was generally well-tolerated. No serious adverse events (SAEs) or deaths were reported, and no subject experienced an adverse event (AE) that resulted in discontinuation. All these events were assessed as unrelated to VGR-R01. Retinal detachment was the most frequently reported AEs related to surgical procedure (n=2) and steroid-associated elevated white blood cell counts (n=3). Most events were mild in severity, and resolved without need for medical intervention. **Conclusion:** Subretinal gene therapy with VGR-R01 demonstrated a good safety profile and resulted in rapid improvement in functional vision in all participants with BCD. With more data being collected, we will continue to assess the effect of VGR-R01. A separate multicenter, open-label phase 1 study of VGR-R01 in patients with BCD is ongoing (NCT05694598).

804 Phase I/II Trial of MUC1*-CAR-1XX T Cells

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Background and Aim: Previous attempts to therapeutically target MUC1 failed because they targeted the tandem repeat domain of full-length MUC1, which is shed from tumor cells. More recently, CARs have incorporated antibodies, such as 5E5, that recognize trapped glycans that deposit on sites for O-linked glycosylation. However, these also target the tandem repeat domain of full-length MUC1, which alone contains sites for O-linked glycosylation. It is the transmembrane cleavage product, called MUC1* (muk 1 star), that is the growth factor receptor form of MUC1. Cleavage of MUC1 by specific, tumor-associated enzymes leads to the unfolding of the MUC1* portion, which reveals a previously hidden binding site for an onco-embryonic growth factor. The aim was to develop an antibody that recognizes this cryptic binding site, which is only revealed on cancer cells after MUC1 is cleaved to MUC1*, and incorporate it into a next generation CAR T cell. Other antibodies that bind to a linear epitope on a MUC1 cleavage product such as MUC1-C will also bind to that same linear epitope on normal, full-length MUC1. Methods: We developed a panel of monoclonal antibodies that bind to a cryptic site that is only revealed after MUC1 is cleaved to the growth factor receptor form called MUC1*. Enzymes that are known predictors of poor prognosis in solid tumor cancers cleave MUC1 to MUC1*, creating a specific conformational epitope that does not exist in full-length MUC1. Antibodies that bind to a linear epitope will also bind to full-length MUC1 so will have on-target/off-tumor toxicities. We incorporated an antibody that binds to the cryptic site on MUC1* into both a standard CAR T, huMNC2-CAR44, and a CAR-1XX, called huMNC2-CAR22. The "1XX" mutations, consist of 4 Tyrosine to Phenylalanine mutations in ITAMs 2 and 3 of CD3z, but leave the ITAM 1 intact. These mutations prevent Tyrosine phosphorylation and thus slow signaling. Results: In animals, huMNC2-CAR22 outperformed huMNC2-CAR44 on two major fronts: persistence and ability to kill low antigen expressing cancer cells while still having no effect of normal MUC1 expressing cells. We expect huMNC2-CAR22 to outperform huMNC2-CAR44 in a human trial for metastatic breast cancers, which is now open. Having said that, 7 patients have been treated to date with huMNC2-CAR44. Efficacy was measured even at low dose with best response seen at Day 28. 4 of the 7 patients had Stable Disease and 1 patient, with bone metastasis, had a Partial Metabolic Response. To date, there is no evidence of on-target/off-tumor toxicities. We expect to have patient results before ASGCT 2023. A CD19-CAR-1XX trial of 16 patients (Park et al ASH 2022 Abstract 163) reported efficacy at reduced dose, compared to the CD19-CAR-CD3z-wt, greatly increased persistence and reduced CRS incidents and severity. We expect huMNC2-CAR22, aka MUC1*-CAR-1XX, to show greater efficacy in patients because of prolonged persistence, ability to kill low MUC1* expressing cells, with increased safety due to reduction in CRS. Conclusion: Results to date of huMNC2-CAR44 Phase I/II trial validate this new target, MUC1*, for cellular therapies for the treatment of solid tumor cancers. We expect huMNC2-CAR22 to be a significant improvement over its predecessor, huMNC2-CAR44, based on its greatly increased persistence, reduced CRS in human as well as its ability to kill low antigen expressing cancer cells.

805 Biomarkers Reveal Improved Red Cell Health Following Hematopoietic Stem Cell Transplantation (HSCT) for SCD

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Introduction: Despite new therapeutic options, sustainable improvement in clinical outcomes and quality of life in sickle cell disease (SCD) remain elusive. Allogeneic hematopoietic stem cell transplant (HSCT) is the most widely available curative option for SCD. Its efficacy is assessed by frequency of pain crises, hemoglobin (Hb) response, markers of hemolysis, and level of engraftment. Normalization of red blood cell (RBC) health is a fundamental goal of successful HSCT; however, the normalization of RBC health in response to HSCT has not been clearly established. Mechanical fragility (MF) and flow adhesion of whole blood to vascular cell adhesion molecule (FA-WB-VCAM) and P-selectin (FA-WB-Psel) are standardized blood-based biomarkers developed in our lab to assess RBC membrane stability and adhesive properties respectively. The objective of this study was to determine if MF, FA-WB-VCAM, and FA-WB-Psel can monitor the normalization of RBC function in individuals with SCD post-HSCT. Methods: SCD subjects were enrolled in NIH haploidentical HSCT protocol 17-H-0069. Blood samples from 3 of 10 planned subjects were collected twice before (baseline) and 3, 6, and 12 months after HSCT. FA-WB-VCAM and FA-WB-Psel were performed at 1.0 dyne/cm^2 using a BioFlux 1000Z microfluidic well-plate system. Adherent cells were quantified and reported as cells/mm^2. MF was assessed with a proprietary system comprising an electromagnetic bead mill and fiberoptic spectrophotometry detection for non-invasive measurement of mechanically-induced RBC hemolysis. Results: Prior to HSCT, all SCD subjects experienced recurrent vaso-occlusive crises, chronic pain, and transfusion-associated iron overload. Average pre-HSCT baselines for FA-WB-VCAM and FA-WB-Psel (470±290 cells/mm^2 and 74±93 cells/mm^2, respectively; n=3) were elevated compared to the previously established high risk threshold for FA-WB-VCAM (408 cells/mm^2) and FA-WB-Psel (46 cells/mm^2). The MF index at 10 minutes (MFI10) was significantly elevated for one patient at 7.38 compared to high risk MF threshold at 7.25 in SCD; it was normal for the other two patients (5.05 and 5.85). Full donor chimerism was observed as early as 3 months post-HSCT. Progressive decrease of pain and opioid use was observed for one patient (at 6- and 12-months post-HSCT); this was not conclusive for other patients at 3 and 6 months. While LDH did not show improvement, bilirubin declined significantly (<0.5 mg/dL post-HSCT for all patients). Average RBC MF decreased 16% while

Hb increased 47% to 13.5g/dL at 12 months. At 6 months, both FA-WB-VCAM and FA-WB-Psel decreased 95% to 25 cells/mm^2 and 79% to 22 cells/mm^2, respectively (See Fig. 1). Conclusions: RBC health objectively normalized following HSCT, as reflected in FA-WB-VCAM, FA-WB-Psel, and RBC MF in the range of AA RBCs. Longer follow-up is necessary to establish clinical improvement, although the pain reduction and decrease in opioid use observed in one of the patients is encouraging. These data suggest FA-WB-VCAM, FA-WB-Psel, and MF may be objective assessment tools of the normalization of RBC health following HSCT.

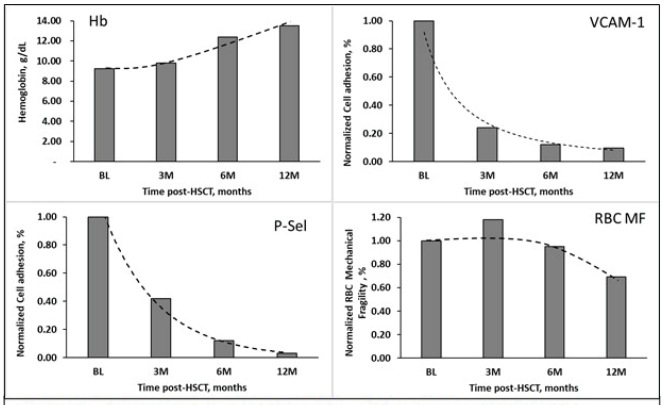

Figure 1. Average changes in Hb and metrics of blood cell function (normalized to individual baseline levels) before and after HSCT. Changes within patients were significant at p-values <0.05.

806 Preliminary Results of Follistatin 344 Episomal Plasma Gene-Therapy in Adipose Fat in Human Patients

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Follistatin 344 is a selective myostatin inhibitor which has shown promise as a target for gene-therapies to treat muscular dystrophy. In this work, an episomal plasmid based vector was developed for the expression of Follistatin 344 for extend periods of time over canonical plasmids. This Follistatin 344 expressing plasmid vector was administered to 50 patients at a dosage of 50 µg via injection into adipose fat tissue. The mechanism of transfection was DNA complexed with Linear Polyethylenimine Hydrochloride. Follistatin and Myostatin concentrations were determined via sandwich type Elisa performed on patient serum. DEXA scan was used to monitor body composition (fat, bone and lean body mass). Triglycerides, HDL, LDL and total cholesterol were monitored via lipid panels. Diabetic markers such as glucose, hemoglobin A1C, insulin and IGF were monitored for insulin sensitivity. Inflammation markers such as high-sensitivity C-reactive protein and secondary hormonal proteins such as luteinizing hormone and follicle stimulating hormone were additionally monitored in the battery of tests. Epigenetic and Glycomic analyses were performed via TruDiagnostic and GlycanAge, respectively.

807 RGX-121 Gene Therapy for the Treatment of Neuronopathic Mucopolysaccharidosis Type II (MPS II): Interim Analysis of Data from the First in Human Study

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MPS II is an x-linked lysosomal storage disease caused by deficiency of iduronate-2-sulfatase (I2S) leading to accumulation of glycosaminoglycans (GAGs) in tissues. Neuronopathic MPS II results in irreversible neurodevelopmental decline not addressed by intravenously administered enzyme replacement therapy. RGX-121, a recombinant adeno-associated virus serotype 9 capsid containing a human iduronate-2-sulfatase expression cassette (AAV9.CB7.hIDS), administered to the central nervous system (CNS) may provide a permanent source of secreted I2S, potentially correcting neurologic and systemic disease manifestations. CAMPSIITE™ is a phase I/II/III, open-label trial enrolling boys 4 months up to five years of age with neuronopathic MPS II (NCT03566043). Participants receive one image-guided RGX-121 injection to the CNS with follow-up through 104 weeks and are encouraged to enroll into a long-term, follow-up study for a total of 5 years. Assessments include safety and tolerability; measurements of CSF, plasma, and urine biomarkers; cognition, language, and motor neurodevelopmental scales; and imaging. As of August 1, 2022, 14 participants were enrolled in the phase I/II portion of CAMPSIITE in 3 dose cohorts ($1.3x10^{10}$, $6.5x10^{10}$, and $2.9x10^{11}$ genome copies/gram brain mass). RGX-121 was reported to be well-tolerated with no drug-related serious adverse events. Time of post-administration follow-up ranged from 8 weeks to 2 years. CSF GAGs, which are elevated in neuronopathic MPS II disease, showed dose-dependent reductions (with D2S6 levels approaching normal levels in cohort 3). As of December 20, 2021, interim neurodevelopmental assessments demonstrated CNS activity up to 2 years after RGX-121 administration. Additionally, there was evidence of systemic enzyme expression and biomarker activity after CNS RGX-121 administration. Updated interim results from this trial will be presented. RGX-121 has the potential to provide sustained CNS clinical outcomes and additional systemic effects in MPS II patients.

808 Optogenetic Therapy with MCO-010 for Vision Restoration in Patients with Severe Sight Loss Due to Retinitis Pigmentosa: The Phase 2b RESTORE Study

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RP is a disorder caused by mutations in more than 80 different genes, resulting in progressive retinal degeneration of the photoreceptors and RPE leading to vision loss. Ophthalmic optogenetics involves the expression of transgenes encoding light sensitive proteins or opsins into the surviving retinal cells to make them directly sensitive to light. Optogenetic therapies work independently of the genetic mutation or cause of retinal degeneration and do not require RPE or photoreceptor viability, offering the potential for vision restoration even in the later stages of retinal degenerative diseases. MCO-010 is a Multi-Characteristic Opsin (MCO), delivered via an intravitreal (IVT) injection as an adeno-associated virus type 2 (AAV2) gene therapy vector with mGluR6 promoter-enhancer for ON retinal bipolar cell expression and mCherry as stabilizer-enhancer and reporter. The MCO-010 fusion protein is highly light sensitive across the entire color bandwidth of visual light with fast kinetics to minimize blur, maximizing each photon to potentially enable high-quality vision without the need for external stimulation devices.

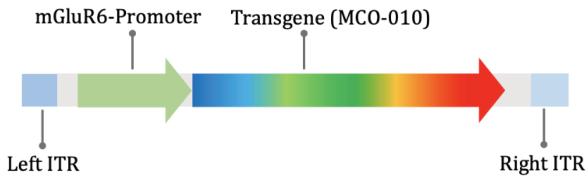

Figure 1. MCO-010 Vector Design. All 11 patients with advanced RP in a phase 1/2a open label study demonstrated improvement in vision following MCO-010 IVT injection. The therapy was well tolerated. The efficacy and safety of MCO-010 in RP patients with severe vision loss has been further assessed in the Phase 2b RESTORE study (NCT04945772). RESTORE is a 2-year, randomized, double-masked, sham-controlled multicenter clinical trial evaluating 2 dose levels of IVT MCO-010 (0.9E11 and 1.2E11 gc/eye) in advanced RP. Inclusion criteria included diagnosis of advanced RP, acuity worse than 1.9 LogMAR for study eye and presence of inner retinal cells on OCT. The primary outcome measure is change from baseline in vision-guided mobility using the Multi-Luminance Y-Mobility Test (MLYMT) at 52 weeks as assessed by central, masked graders. Other vision function tests include change in acuity and shape discrimination assessed by the low-vision multi-parameter test (LVMPT). 27 patients with advanced RP were enrolled, with the final patient randomized in February 2022. At screening, the mean age was 56 years (range 23-83), and mean study eye visual acuity was 2.2 LogMAR (range 1.95-2.25, CF/HM vision). As of February 1, 2023, 24/27 subjects had completed their week 52 study visit with no serious ocular adverse events or drug-related systemic adverse events reported. Primary efficacy and safety 52-week data for all subjects will be presented. MCO-010 is a gene agnostic optogenetic therapy designed for vision restoration in patients with severe sight loss due to outer retinal degeneration. Delivered via a single IVT injection, MCO-010 targets bipolar cells for ambient light sensitization. Efficacy and safety data from the RESTORE 2b trial will provide further evidence on the benefit/risk profile of MCO-010 including its potential to improve vision-guided mobility in patients with advanced RP.

809 Regulatory Pathway for Gene Therapy for Rare Diseases: An Illustrative Example from the PaVe-GT Platform Program

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The Platform Vector Gene Therapy (PaVe-GT) program is an initiative started by a multi-disciplinary group of collaborators to develop adeno-associated virus (AAV) gene therapies for four rare diseases, which as a group, are relatively common but lack effective therapies. With approximately 10,000 rare diseases that affect 25 to 30 million people in the United States and less than 10% of these diseases having an approved treatment option, there is still much work to be done. Addressing the need for therapies for rare diseases under a unified platform, such as PaVe-GT, may serve as a paradigm for reducing process redundancies and increasing efficiencies in the preclinical, regulatory, and clinical activities to broadly enable the development of gene therapies for rare diseases. Here, we showcase the regulatory strategy for the PaVe-GT program's first indication. AAV9-hPCCA gene therapy is being developed to treat propionic acidemia resulting from a deficiency of propionyl-CoA carboxylase, alpha subunit (PCCA). The product's regulatory pathway has thus far progressed successfully through an **INTERACT** (or **IN**itial **T**argeted **E**ngagement for **R**egulatory **A**dvice on **C**BER Produc**T**s) meeting with the FDA and is currently in the pre-IND planning phase. In addition, this gene therapy project has also received two valuable designations: Orphan Drug (ODD) and Rare Pediatric Disease (RPDD), from the Food and Drug Administration (FDA). The ODD and RPDD programs provide sponsors with financial incentives for research and development of drugs for rare diseases. Lessons learned from our regulatory activities will be presented. In conclusion, the knowledge achieved by advancing PaVe-GT program's first product through critical regulatory milestones will be applied to the other three indications under development, with the goal of reducing process redundancies and increasing efficiencies. PaVe-GT represents a strategy for accelerating therapeutic development for rare diseases that can be disseminated to other AAV gene therapy efforts.

Thursday Poster Session

810 Deleterious Genetic Variants in the AAVR Universal Receptor (KIAA0319L) with the Potential to Limit AAV-Mediated Gene Expression

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Clinical studies with adenoassociated virus (AAV) mediated gene transfer demonstrate effective, persistent expression of the AAV transgene, but with significant variability to gene expression among recipients, despite identical doses. One unexplored contributor to this variability could be genetic variants in genes responsible for AAV interactions with target cells. As an initial assessment of this concept, we hypothesized that there may be genetic variation in AAVR (KIAA0319L), a 164 kb gene on chromosome 1 that codes for a type I transmembrane protein that serves as the primary receptor for many AAV serotypes. Human variants of AAVR were assessed in 3 whole genome databases: African/African-American (20,744 genomes, gnomAD), European (34,029 genomes, gnomAD), and Qatari (14,669 genomes, Qatari Genome Project database). Compared to the reference genome, the identified variants (African, European, Qatari) included: total variants (410, 508, 5713); known variants (364, 451, 3613); novel variants (46, 57, 2100); rare variants (allele frequency <0.01; 396, 502, 5194); and common variants (allele frequency ≥0.01; 14, 6, 19). The AAVR variants were classified as deleterious (loss of function) based on one of the following three criteria: (1) high impact loss of function (frameshift, stop or start lost, transcript amplification, stop gained, splice donor or acceptor variants, transcript ablation; African, European, Qatari: 5, 13, 4); (2) moderate impact loss of function (in-frame insertion or deletion, missense, protein-altering, regulatory region ablation; African, European, Qatari: 122, 154, 48); or (3) loss of function using "Combined Annotative Dependent Depletion" (CADD) and "Sorting Intolerant from Tolerant" (SIFT) analyses with the criteria of theoretical dysfunction if the CADD score was ≥20 and if the variant was classified as "intolerant" by SIFT analysis (SIFT score ≤0.05; African, European, Qatari: 51, 88, 34). The list of deleterious variants was then cross-checked with ClinVar and any variant previously described as either "benign" or "likely benign" in ClinVar was removed from the list. The final number of candidate deleterious loss of function AAVR variants were African n=45, European 79 and Qatari 20. Examples of loss of function AAVR variants included: (1) missense Lys3Thr (CADD 22.8, SIFT 0, allele frequency 0.03 in Qatari, rare in African and European); (2) missense Pro302Gln (CADD 23.1, SIFT 0.019, rare in African and Qatari); and (3) missense Ala563Val (CADD 27, SIFT 0.012, rare in European). While homozygosity of these deleterious variants likely will have a significant impact of the effectiveness of AAV gene expression, future studies will be needed to assess the impact of heterozygosity. In summary, we have identified potentially deleterious human genetic variants in AAVR, a gene coding for a critical step in gene transfer for many AAV serotypes. We suggest that this, as well as possible deleterious genetic variants in other genes coding for cellular components critical for AAV gene transfer, may play a role in variations in levels of gene expression observed in human gene therapy. Identification of these genetic variants relevant to effective gene transfer should contribute to "precision gene therapy."

811 Cas13d Multi-Targeting Efficiently Targets Sense and Antisense HRE Containing Toxic RNAs and poly-GP DPR in C9ALS Patient Cells and in C9-BAC500 Mouse Model

Daniela Martino Roth, Jeannie Chew, Hank Bradford, Takahiro Tadokoro, Akiyo Ogawa, Shawn Lee, Joshua Fong, Gustavo Villegas, Claire Geddes, Vincent Luczak, Haydee Gutierrez, Ronald Torres, Nandini Narayan, Sheik Pasha, Justin Truong, Anindita Sarkar, Greg Nachtrab, John E. Leonard, Ranjan Batra

Locanabio, San Diego, CA

Hexanucleotide repeat expansion (HRE) in the first intron of C9ORF72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontal temporal dementia (FTD). Bidirectional transcription at the C9ORF72 repeat locus produces both sense GGGGCC (G4C2) and antisense CCCCGG (C4G2) containing toxic RNAs. Studies to date have elucidated multiple pathogenic mechanisms, including RNA gain-of-function of both sense and antisense HRE transcripts leading to formation of nuclear RNA foci and dipeptide repeat protein (DPR) aggregates generated by RAN translation. CRISPR/Cas13d is a type VI programmable nuclease with the ability to process guide-RNA arrays and target multiple RNAs for degradation. We developed a multi-targeting (MT) Cas13d construct to knockdown both sense and antisense transcripts of the C9ORF72 HRE and packaged it in a single AAV9 vector. Cas13d-MT reduced both G4C2 and C4G2 RNA Foci, sense and antisense repeat containing transcripts, and poly-GP DPR levels in C9ALS patient derived fibroblasts. Invivo studies with AAV9 packaged Cas13d-MT delivered via subpial (spinal cord) or intrastriatal injection in C9BAC-500 mice showed significant reduction of both sense and antisense HRE containing RNAs, 50-60% decrease in poly-GP DPR, and maintenance of total C9ORF72 mRNA levels. We observed sustained expression of the transgene and no signs of toxicity in-life or with histopathology at 6-8 weeks post treatment in both spinal cord and brain. Overall, our multi-targeting strategy is safe and efficacious in targeting two disease-causing RNAs simultaneously in C9ALS *in vitro* and *in vivo* models.

812 Single Dose Cocktail of AAV Vectors Coding for Peanut Allergens Prevents Peanut Allergy

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Introduction: Food allergy (FA) is a health problem with an increased incidence in children. FA results from an abnormal response in gut immune tolerance that leads to the activation of B cells and immunoglobulin E (IgE) class switching. Sensitization to food allergens in humans can result from different exposure routes such as the gut, respiratory tract or even the skin. The most common food allergens are

peanut (PE), milk, eggs, and tree nuts. Immunotherapies for patients with FA have shown some success in limiting allergic responses however, these approaches require lengthy protocols with repeated allergen dosing and patients can relapse following discontinuation of treatment. Following up on our previously published studies in a single allergen food allergy model, we hypothesized that we could prevent PE allergy with a single dose of adeno-associated vectors (AAV) expressing the most common PE allergens (Ara h 1, 2, 3, and 6) in the liver. Methods: 6-week-old heterozygous flaky tail ($FT^{+/-}$) female mice were injected with 10^{11} vg of a single AAV8 vector expressing Ara h 1, 2, 3, or 6, or a cocktail of all four vectors. Four weeks later, mice were bled to determine the Ara h protein expression in plasma by ELISA. Groups showing expression of Ara h proteins in plasma (animals injected with Ara h 2, 6, and AAV cocktail) and a non-AAV treated mice as control group were sensitized by epicutaneous administration (8 doses) of *Alternaria alternata* protein extract followed by PE extract. After sensitization, mice were bled, and anaphylaxis was assessed by a reduction in the core body temperature (CBT) and an increase in the symptom score (SS) scale (0: No symptoms; 1: scratching nose/head; 2: reduced activity; 3: labored respiration; 4: no activity after prodding; 5: death) measured every 15 minutes after an intraperitoneal PE injection. Finally, livers were collected to determine the Ara h protein expression and PE-specific IgE and IgG1 titers were determined in plasma by ELISA. Results: In the single AAV8-Ara h treated groups we detected Ara h 2 and 6 expression in plasma and Ara h 1, 2, 3, and 6 in the liver. A comparison between single AAV8-Ara h and AAV cocktail treated animals showed higher expression of Ara h proteins in those groups injected with a single AAV in Ara h 1 and 3 in liver (1126±56.9 vs 198.7±48.5 ng/mg of liver protein and 1.1±0.1 vs 0.6±0.1 ng/mg of liver protein for Ara h 1 and 3, respectively), Ara h 2 in plasma (44.9±6.4 vs 38.3±1.7 ng/ml), and Ara h 6 in liver and plasma (148.3±6.1 ng/mg of liver protein and 78.9±2.7 ng/ml vs 112.3±6.2 ng/mg of liver protein and 66.1±3.2 ng/ml). Nonetheless, higher expression of Ara h 2 was detected in the liver of mice receiving the AAV cocktail compared with the AAV8-Ara h 2 alone (5.7±0.7 vs 1.6±0.5 ng/mg of liver protein). After sensitization, only mice pretreated with the AAV cocktail showed a significant reduction in hypothermia (0.4°C ±0.2) and SS (0.6±0.2) compared with those treated with AAV8-Ara h 2 (-3.5°C ±1.2 and 2.7±0.2) and non-AAV group (-4.6°C ±0.31 °C and 2.7±0.3). A slight reduction in hypothermia (-2.4°C ±1) and SS (1.7±0.4) was observed in mice treated with AAV8-Ara h 6 alone compared with Ara h 2 and non-treated groups. Protection from PE allergy was associated with a reduction in PE-specific IgG1 and IgE levels. Conclusions: We demonstrated that a single dose of a cocktail containing AAV vectors coding for the liver expression of the four major PE allergens (Ara h 1, 2, 3, and 6) can protect from skin sensitization in a FA model. The reduction in the expression of the Ara h proteins in the AAV cocktail treated group suggests a role for receptor competition for hepatocyte transduction, but this does not compromise the protective effect of the therapy. Although treatment with AAV8-Ara h 6 alone showed a partial reduction of allergic symptoms, complete protection from PE allergy requires simultaneous expression of multiple PE allergens.

813 Engineering a Multi-Cytokine Backpack Platform for Manufacturing Functionally Improved CAR T Cell Products

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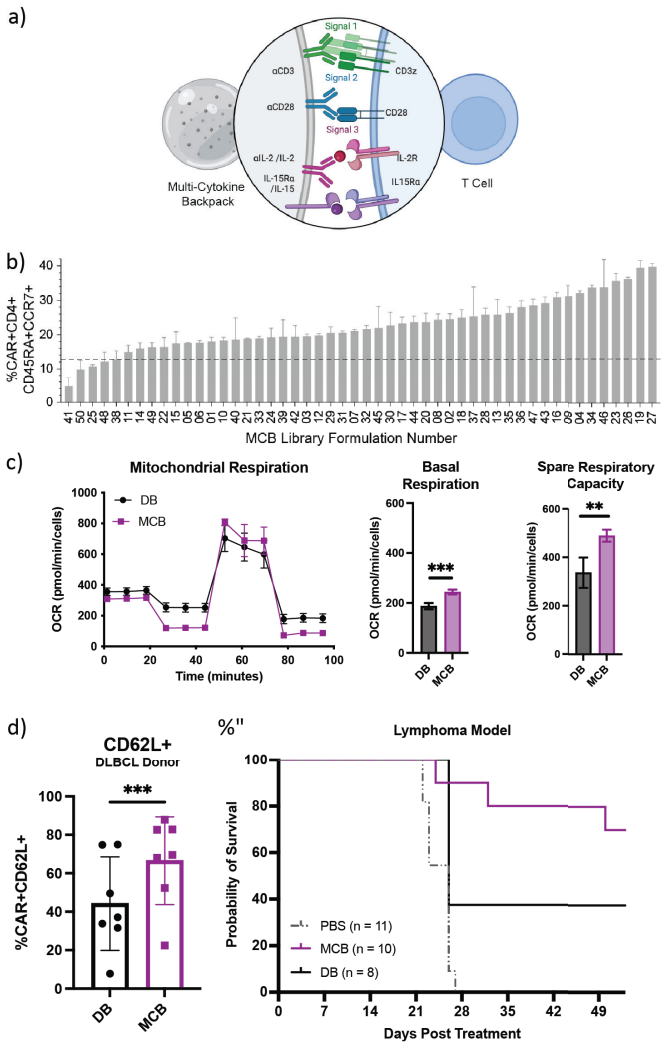
Despite the remarkable clinical efficacy of chimeric antigen receptor (CAR) T cell in diffuse large B cell lymphoma (DLBCL), only ~60% of treated patients do not benefit either due to lack of initial response or a short duration of response to therapy. Emerging research demonstrates that the initial phenotype of the CAR T cell product after manufacturing, specifically less-differentiated phenotypes, can greatly impact therapeutic outcomes. Reagents that consistently manufacture memory phenotypes within CAR T cell products could significantly improve clinical outcomes in DLBCL. We have developed a modular multi-cytokine backpack (MCB) platform which provides the signals necessary for activation, costimulation, and cytokine support during CAR T cell manufacturing in a single "all-in-one" reagent (Fig 1a). This platform affords a high degree of flexibility that allows for the assembly and screening of compositionally diverse MCB libraries to identify formulations tailored to promote specific phenotypes. For proof-of-concept of this technology, we used multi-dimensional flow cytometry to screen MCB-manufactured CD19-directed CAR T cells for the highest proportions of less-differentiated phenotypes (CD45RA+CCR7+, Fig 1b). Statistical clustering of MCB library CAR T cell phenotypes identified top candidates for further experimentation to compare to standard DynaBeads with exogenous IL-2 (DB). CAR T cells generated from DLBCL patient samples showed significantly greater proportions of less-differentiated CD62L+ cells present in MCB- compared to DB-manufactured products ($p=0.005$, Fig 1d). Evaluation of MCB-manufactured healthy donor (HD) CAR T cells by mass cytometry and subsequent FlowSOM analysis identified central memory clusters of MCB-manufactured HD CD8+ CAR T cells that significantly express more TCF1 ($p=0.014$) and CD44 ($p=0.002$). Seahorse mitochondrial stress assays revealed that antigen-stimulated MCB-manufactured CAR T cells had increased basal respiration ($p=0.0005$) and spare respiratory capacity ($p=0.004$, Fig 1c). In vivo, all formulations of MCB-manufactured CAR T cells reduce tumor burden and enhance survival benefits in lymphoma ($p<0.0001$, Fig 1e) and ovarian cancer ($p=0.008$) models. MCB-manufactured CAR T-treated mice had more hCD3+ cells in peripheral blood on day 21. Long-term surviving mice treated with MCB-manufactured CAR T cells were rechallenged on day 188 and had significantly less tumor burden ($p = 0.015$) and improved overall survival ($p=0.046$). In summary, we have engineered a powerful platform capable of screening "all-in-one" manufacturing reagents without the need for exogenous cytokine supplementation that can provide all the necessary signals to generate CAR T cell products with improved phenotypes. Through our platform, we identified MCB formulations that resulted in CAR T cell products with greater proportions of memory phenotypes, evidence of self-renewal and metabolic fitness. Importantly, MCB-

manufactured CAR T cells reduce tumor burden and enhance overall survival in both hematologic and solid malignancies, with improved in vivo persistence of functional CAR T cells.



814 *In Vivo* FiCAT Programmable Gene Writing Platform Based on Lipid Nanoparticles Delivery System

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In our previous work we developed a robust gene delivery tool (FiCAT), combining the precision of Cas9 DNA scanning and targeting DNA with an engineered piggyBac transposase with donor DNA processing and transfer capacity. Here, with the aim to demonstrate FiCAT activity *in vivo*, we performed precise gene delivery to the liver using non-viral carriers that trigger less immunological responses compared to viral-based approaches. At this point, lipid nanoparticles (LNPs) have been extensively validated both pre-clinically and clinically for the delivery of different nucleic acid cargos. We first optimized both, DNA and RNA LNP formulations for *in vivo* nucleic acid delivery. With DNA-based nanoparticles, a biodistribution assessment was also performed to ensure that transgene expression was restricted in the liver. After optimizing long mRNA encapsulation and delivery with FiCAT, we precisely inserted the DNA transposon codifying our gene of interest by co-delivering FiCAT machinery to mice liver targeting Rosa26 genomic safe harbor. Precise gene writing was confirmed by direct sequencing of junction PCR in isolated livers of mice 4 weeks after LNPs systemic injection. Here, we aim to safely deliver DNA and mRNA cargos via LNPs *in vivo* thanks to our gene writing machinery (FiCAT). This precise manner of inserting entire payloads in a genome region of interest opens a window for the treatment of several genetic disorders, making an important breakthrough in gene therapy field.

815 Frequent Aneuploidy in Primary Human T Cells after CRISPR-Cas9 Cleavage

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Multiple clinical trials of allogeneic T cell therapy use site-specific nucleases to disrupt T cell receptor (TCR) and other genes. In this study, using single-cell RNA sequencing, we investigated genome editing outcomes in primary human T cells transfected with CRISPR-Cas9 and guide RNAs targeting genes for TCR chains and programmed cell death protein 1. Four days after transfection, we found a loss of chromosome 14, harboring the TCRα locus, in up to 9% of the cells and a chromosome 14 gain in up to 1.4% of the cells. Chromosome 7, harboring the TCRβ locus, was truncated in 9.9% of the cells. Aberrations were validated using fluorescence in situ hybridization and digital droplet PCR. Aneuploidy was associated with reduced proliferation, induced p53 activation and cell death. However, at 11 days after transfection, 0.9% of T cells still had a chromosome 14 loss. Aneuploidy and chromosomal truncations are, thus, frequent outcomes of CRISPR-Cas9 cleavage that should be monitored and minimized in clinical protocols.

816 Suppression of Neurological Deterioration in the APP1.PSEN1/APOE4 Murine Model of Alzheimer's Disease by AAV-Mediated Expression of APOE2 Christchurch

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Humans homozygous for the APOE4 mutation (C112R) have a 15-fold greater risk of developing Alzheimer's disease compared to those with the APOE3 allele, while the APOE2 allele (R158C) is protective

against Alzheimer's disease. The report (Nature Med 2019; 25:1680) describing a subject homozygous for APOE3 with the Christchurch mutation (R136S) without cognitive impairment despite carrying the autosomal dominant PSEN E280A allele suggests a protective role of the APOE Christchurch mutation against Alzheimer's disease. In this study, we tested the hypothesis that AAVrh.10-mediated expression of the combined human APOE2 allele with the Christchurch mutation (AAVrh.10hAPOE2Ch), will effectively protect against the development of Alzheimer's disease in the APP.PSEN1/TRE4 amyloid plaque mouse model which has a humanized APOE4 gene, an amyloid plaque promoting presenilin mutation and a mutant amyloid precursor protein. When untreated, these mice develop the morphologic, biochemical and behavioral manifestations of Alzheimer's disease. The AAVrh.10APOE2Ch vector was administered to the hippocampus ($2x10^{10}$ genome copies) of APP.PSEN/APOE4 mice at age 2.5 months with assessment at 5.5 months. Control animals were administered PBS. The AAVrh.10hAPOE2Ch cohort had a marked increase in hippocampal human APOE protein (10.5±1.5 ng/μg compared to PBS 0.15 ± 0.03 cohort, $p<0.001$). AAVrh.10hAPOE2Ch administration reduced the levels of both soluble and insoluble amyloid peptide β42 by 77.60% and 81.90%, and soluble amyloid peptide β40 by 55.8% and 64.2%, respectively compared to the PBS cohort ($p<0.001$ all comparisons). Immunohistochemical staining of the hippocampus for β-amyloid demonstrated a decrease in the number and size of amyloid plaques following AAVrh.10hAPOE2Ch administration compared to PBS controls ($p<0.001$). Fluoro-Jade staining for neuronal degeneration in mutant mice was decreased from 40.8±3.2% in PBS mice to 21.4±2.2% in AAVrh.10hAPOE2Ch treated mice ($p<0.001$). Markers of astrogliosis and microgliosis showed a AAVrh.10hAPOE2Ch dependent decrease in GFAP and Iba1 positive cells, decreasing from 30.6±1.9% to 19.1±3.3% and 32.3±3.6% to 11.6±1.1% ($p=0.005$, $p<0.001$, respectively). Prior to sacrifice, the mice were assessed by 3 behavioral sensorimotor assays. Compared to PBS, AAVrh.10hAPOE2Ch treatment: (1) increased the number of alternations in the Y maze (9.25±1.19 compared to 5.50±0.63 in the PBS group, $p<0.001$); (2) improved the novel object discrimination index 0.20±0.09 compared to -0.17±0.06 in PBS ($p<0.007$); and (3) decreased escape latency in the Barnes maze test to 25.0±4.5 seconds compared to 89.7±2.9 seconds in the PBS cohort ($p<0.001$), all indications of improved cognition and memory in mice. In conclusion, intrahippocampal delivery of AAVrh.10 expressing human APOE2 with the Christchurch variant improves morphologic, biochemical and behavioral disease parameters in the amyloid-driven Alzheimer's mouse.

817 Our Proprietary Manufacturing Process, mAAVRx, Improves Quantity and Quality of scAAV Vectors

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Unlike traditional single strand AAV (ssAAV) vectors, self-complementary AAV (scAAV) vectors can produce self-complementary intramolecular dsDNA genomes without depending on the host cell DNA polymerase machinery. This can help to achieve faster, and higher levels of gene expression compared to ssAAV vectors. However, the manufacturing yields and the full-to-empty capsid ratio tend to be more challenging for scAAV than ssAAV. Previously, we reported that our proprietary manufacturing process, mAAVRx, increased vector titers and purity for ssAAV across different serotypes. Here, we describe the manufacturing and characterization of a scAAV vector to understand whether mAAVRx could improve the yield and quality of the produced scAAV vectors. We used AAV9 as the capsid serotype and SMN1 as the transgene in a case study to produce scAAV. The molar ratio for mAAVRx two-plasmid system was first optimized. Compared to the traditional three-plasmid system, mAAVRx improved the crude harvest titer by 2-fold for scAAV9-SMN1, reaching crude harvest titers between 4e11 vg/mL and 8e11 vg/mL depending on the HEK293 cell line used. We further evaluated the quality of the vectors. Vector genome analysis by alkaline gel showed one single band of the correct target genome size indicating the integrity of the packaged AAV genome. mAAVRx produced a higher percentage of full capsids compared to the traditional three-plasmid system before and after full capsid enrichment. The full capsid-percentage, measured by AEX-HPLC, was 25% with mAAVRx and 19% with the three-plasmid system. After cesium chloride ultracentrifugation purification, mAAVRx yielded 95% full capsids whereas a traditional three-plasmid system yielded 78%. In addition, the vector potency was evaluated in vivo for each production system. Wild-type mice were injected intravenously with scAAV9-SMN1 at 1E13 vg/kg. Samples from different tissue types including brain, muscle and liver were collected at multiple time points after injection and analyzed for vector copy number and mRNA expression. This result helps to further understand the in vivo potency of the vectors produced by mAAVRx. Our data supported the use of our proprietary manufacturing process, mAAVRx, for the manufacturing of ssAAV and scAAV vectors. mAAVRx showed the ability to increase vector titers and quality attributes such as genome integrity and the percentage of full capsids compared to conventional three-plasmid system.

818 Comparative Assessment of the Efficacy and Safety Associated with Delivery of scAAV-9-CB-GFP via Lumbar Puncture Route to Juvenile Cynomolgus Macaques with and without AAV9 Pre-Existing Immunity

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Pre-existing immunity to the AAV viral capsid represents a major hurdle for AAV-based gene therapeutics. This is due partly to the fact that a substantial portion of the patient population is exposed to wild type AAVs naturally which leads them to develop antibodies against the capsid. In most clinical trials, participants must display antibody titers below a certain cut-off to be enrolled. As the prevalence of pre-existing immunity to AAV increases with age, it presents a unique challenge

for enrolling and treating patients with late/adult-onset neurological diseases. AAV-based intrathecal gene therapies administered to CSF are currently being explored in both preclinical and clinical studies. The role that the presence of pre-existing AAV antibody play in this setting is not clear. The goal of this study is to offer a comparative assessment of efficacy and toxicity markers associated with intrathecal (lumbar puncture) injection of AAV9 vectors in non-human primates (NHP) with and without preexisting immunity to AAV9. Self-complementary AAV2/9- containing a chicken beta -actin promoter driving the expression of green fluorescent protein [GFP] (scAAV9-CB-GFP) was administered via lumbar puncture to juvenile male cynomolgus macaques with and without preexisting anti-AAV9 antibody at a dose of 5 X1013 vector genome per animal and examined for a study duration of 28 days. Central nervous system (CNS) and peripheral tissues were surveyed for vector genome, mRNA, and protein biodistribution as well as for histopathology assessments. Clinical pathology and humoral immune response to the vector capsid and the transgene were also evaluated. Our data reveal that a negative or positive serology to anti-AAV9 antibody prior NHP intrathecal dosing leads to distinct peripheral organ transduction along with different enzyme activities in the liver. We also observed different expression patterns of markers in the CSF that can potentially inform safety and provide a more educated interpretation of efficacy and toxicity findings.

819 E-selectin-Overexpressing Mesenchymal Stem Cell-Based Therapy Reverses Wound Healing Impairments in Ischemic Limbs

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Purpose: Unmodified mesenchymal stem cell (MSC) therapies have failed to demonstrate more than minor clinical benefits for inducing therapeutic angiogenesis and wound healing in patients with chronic limb-threatening ischemia (CLTI), and tissue loss considerably worsens the risk of extremity amputation for these patients. Here we report the effects of MSCs engineered to overexpress E-selectin, a cell-adhesion molecule capable of stimulating neovascularization, using a translational murine model recapitulating hindlimb ischemia and cutaneous tissue loss. **Methods:** MSCs were harvested from bone marrow of 8-10-week-old FVB/Rosa26Sor^{mTmG} donor mice and underwent flow cytometry to confirm Sca-1$^+$/CD29$^+$/CD44$^+$/CD73+/CD105$^+$ expression. MSCs underwent viral transduction with E-selectin-GFP/AAV or GFP/AAV. Surgical ligation of the femoral artery was performed in 12-14-week-old recipient FVB mice followed by a subsequent 4 mm cutaneous wound in the ipsilateral limb and local injection of PBS or 1x10^6 donor GFP$^+$/MSCs or E-selectin$^+$/MSCs (E-sel$^+$/MSC). Wound closure was monitored daily for 7 days, and tissue specimens were harvested. Confocal microscopy and whole-body Dil perfusion were performed to evaluate vascular density of wound tissue. **Results:** Untransduced MSCs do not express E-selectin, and E-sel$^+$/MSCs retained MSC phenotype. Mice receiving E-sel$^+$/MSC treatment (n=10) demonstrated accelerated wound closure compared to GFP$^+$/MSCs (n=10) and PBS (n=10) treated mice at each post-injury day (PID) with the greatest difference observed at PID 5 (94 ± 3% *vs.* 79 ± 10% GFP *vs.* 72 ± 9% PBS, p<0.001) and 7 (98 ± 2% *vs.* 86 ± 8% GFP *vs.* 87 ± 6% PBS; p<0.010). Collagen deposition was more abundant in wounds receiving E-sel$^+$/MSCs (21 ± 4% *vs.* 5 ± 1% GFP *vs.* 9 ± 1% PBS). DiI perfusion at PID 10 demonstrated increased vessel density in wounds treated with E-sel$^+$/MSCs (76 ± 15% *vs.* 29 ± 7% GFP *vs.* 9 ± 5% PBS relative vascular density). E-sel$^+$/MSCs exhibited improved viability with more mTmG$^+$-E-sel$^+$/MSCs (13 ± 3 cells/high powered field (HPF) *vs.* GFP$^+$/MSCs 3 ± 2 cells/HPF *vs.* PBS 0 ± 0 cells/HPF, p<0.010) at PID 7 by immunofluorescent microscopy. **Conclusion:** E-selectin-overexpressing MSCs overcome impairments associated with wound healing in ischemic limbs. These data exhibit the potential role for E-selectin-modified MSCs as a novel cell therapy in future clinical applications for delayed and non-healing wounds in patients with CLTI.

820 Targeting a NIS-Expressing Oncolytic Adenovirus to Colorectal Cancer

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Introduction: Many patients with colorectal cancer will ultimately develop advanced or metastatic disease at some point during their disease course. Unfortunately for some of these individuals, surgical resection and systemic chemotherapy may not effectively treat the cancer. Novel treatments are desperately needed for these individuals and oncolytic adenoviruses (OAd) represent one such innovative treatment approach. Methods: Our group has designed an oncolytic adenovirus backbone structure which utilizes a ΔE3 structure and expresses the sodium iodide symporter (NIS) with each cycle of viral replication (OAd-NIS). Optimal fiber structure, cytocidal effects, and transgene expression were evaluated *in vitro* in multiple human colorectal cancer cell lines. Furthermore, PET/CT imaging was used to demonstrate the ability of the virus to facilitate imaging and radiotracer (^{124}I) uptake in colorectal tumors in an *in vivo* murine model. Results: Previously, our group has reported the use of a NIS-expressing oncolytic adenovirus to facilitate NIS expression and radionuclide uptake in pancreatic cancer and lung cancer models. Here, we utilize the ΔE3 NIS backbone structure and optimize the vector to target colorectal cancer. Multiple adenovirus fibers (Ad5 Wt, Ad 5/3, RGD) were tested *in vitro* to determine which would yield optimal OAd binding, transduction, and replication. In all cell lines tested, the Ad 5/3 fiber was superior. In crystal violet assays, the OAd-NIS virus demonstrated strong cell-killing effects across multiple human colorectal cancer cell lines. Notably, this robust cytocidal effect was present in the OAd-NIS virus which incorporated the Cox2 tissue specific promoter. Following anti-human NIS antibody staining *in vitro*, there was evidence of strong NIS expression in all tested cell lines. Human colorectal cancer xenografts were established in the flanks of nude mice and subsequently treated with the OAd-NIS virus. After ^{124}I administration (via peritoneal injection), the Sophia G8 system was used to acquire PET/CT images. Flank tumors demonstrated strong signal which highlights the ability of the OAd-NIS virus to facilitate NIS expression in the tumors allowing for radionuclide-based imaging for colorectal cancer. Conclusion: The binding and transduction of our OAd-NIS virus in colorectal cancer can be optimized with the use of a chimeric Ad 5/3 fiber. The vector

demonstrates robust cytocidal effects and NIS expression *in vitro* across multiple colorectal cancer cell lines. Strong NIS-transgene expression enables PET/CT imaging with radioactive iodine. Future studies will utilize patient-derived tissues to further evaluate the OAd-NIS in *ex vivo* and *in vivo* models. Additionally, radioiodine ^{131}I will be used in combination with OAd-NIS in murine models to evaluate the oncolytic effects of the combination regimen.

821 Potential Treatment for CMT2S Caused by IGHMBP2 Cryptic Splice Variant with ASO Based Therapeutic

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Charcot-Marie-Tooth disease Type 2S (CMT2S) (OMIM 616155), is a rare autosomal recessive type of CMT, an inherited peripheral neuropathy. Rare variants in immunoglobulin mu-binding protein 2 (IGHMBP2) have been shown to cause CMT Type II. Specifically, they have been shown to result in abnormal RNA processing which likely leads to alpha-motor neuron degeneration. CMT2S is primarily characterized by distal muscle atrophy, weakness with areflexia, and relatively minor sensory involvement. A patient was reported with a suspected diagnosis of CMT. Further evaluations on this patient has confirmed existence of consequential variants within IGHMBP2. Whole genome sequencing has revealed a paternally inherited cryptic splice site variant (inherited non-coding variant (c.1235 + 894 C>A) deep in intron 8). RT-PCR analysis was consistent with activation of the cryptic splice site. Bidirectional sequencing of cDNA derived from the patient's cells identified both ends of the splicing alteration. The abnormal transcript was shown to undergo nonsense-mediated decay (NMD), resulting in haploinsufficiency. The objective of this study was to target IGHMBP2, specifically the cryptic splice site variant, with a novel antisense oligonucleotide (ASO) designed to avoid nonsense-mediated decay and haploinsufficiency. We have obtained the patient's fibroblast cell line and confirmed the variant with whole genome sequencing (WGS). Furthermore, we also confirmed existence of NMD induced by cryptic splicing. We designed an ASO targeting specifically deep in intron 8 (c.1235 + 894 C>A). The ASO was an 19mer targeting the sequence around CACTTCCAC(A)GGGGGAAGA. Analysis of the area of interest led to the design of several ASOs. These were further screened and prioritized based on optimal in silico binding affinity. The designed ASOs were chemically modified by adding a phosphorothioate backbone MOE. CMT Type II patient-derived fibroblast cells underwent ASO treatment (10uM) and incubation for 48h. Successful cellular entry of the ASO was additionally confirmed with flow cytometry and fluorescin labelled ASO (GFP+ 99.8%). Upon treatment with ASO, we have observed a significant increase (~30% increase) of the IGHMBP2 protein levels in the oligo treated samples as compared to control (untreated samples) (WB antibody Sigma SAB2106426). Additionally, qPCR results confirmed increased ratio of restored WT transcript to cryptic exon-containing transcript (~1.3-fold). Furthermore, the ASO has shown to have limited off-target effects in silico. While the improved clinical formulation of the tested ASO may be necessary, the current preclinical data are supportive of being a potential treatment restoring the IGHMBP2 protein levels. An increased number of cases of autosomal recessive Charcot-Marie-Tooth disease Type 2S caused by IGHMBP2 consequential variants are being reported. The N-of-1 precision medicine approach may prove instrumental to the design of treatments for this highly diverse genetic disorder. This case exemplifies the shifting boundary between rapid WGS-based clinical diagnoses available and research capabilities allowing for the design of personalized ASO-based treatments.

822 Integration of a Microfluidic Mechanoporation Device in the Gene-Edited CAR-T Cell Production Workflow

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Introduction: Adaptive cell therapies, including Chimeric Antigen Receptor-modified (CAR) T cell therapy and T Cell Receptor-modified (TCR) T cell therapy, have the potential to provide a curative medicine to oncological diseases, due to their ability to form memory populations that persist for decades. Successful translation of them is enabled by gene editing technologies that provide targeted cytotoxicity to immune cells through the delivery and expression of synthetic receptors required to target tumor antigen and destroy tumor cells. In addition, gene editing can be applied to introduce new functions or disable negative regulators of immune cell killing to generate more potent cell products. As the field of gene engineering progresses, developing of scalable and affordable gene delivery technologies is also required. The current cell therapy products rely on viral vectors to internalize transgenes. The process is costly and labor-intensive, display heterogeneity in their quality attributes, and pose a risk of insertional mutagenesis. Electroporation is a conventional non-viral method to deliver gene modifying cargo has been used in manufacturing of gene edited T cells. Electroporation demonstrated 15-45% gene disruption rate in cGMP manufacturing. However, high delivery efficiency can be accompanied by cell loss, delayed expansion, genetic lesion, and aberrant cellular physiology, which may pose a risk to product safety and functionality. As an alternative, we have developed a microfluidic mechanoporation device capable of delivering large biomolecules (>2,000 KD) to a variety of cell type via volume exchange between the cytosol and surrounding media, which is induced by repeated and rapid compression of cells. This volume exchange for cell transfection (VECT) device achieved superior cell viability and proliferation without sacrificing the delivery efficiency. In this study, we aim to test compatibility of VECT device integrated in a CD5-knockout CAR-T cell manufacturing workflow. Result: We first evaluated the cell biomechanical responses of unactivated and 1 activated primary T cells. T cells were found to expand in size and grow softer over 3-days of activation. We next optimized microfluidic channel designs for processing T cells by considering a number of device parameters. Under tested conditions, we could show >90% delivery of 500KD dextran to non-activated and 1-day activated T cells with over 80% viability. We then demonstrated effective production of CD5-knockout CAR T cells using a combined microfluidics and viral transduction approach. We performed CRISPR-based knockout of the CD5 locus in 1-day activated T cells and achieved 65% knockout. Edited T cells were transduced with a CD19 CAR in a 20hr coincubation with a lentiviral vector. After 10-day expansion, processed cells result in a 4-fold increase in gene edited CD5-knockout CAR T cells compared to a workflow using electroporation. We

found that rapid compressions in VECT devices did not impair the transduction efficiency (35% in VECT processed and 28% in control population). Processed T cells also show similar composition in stem memory T cells. In a killing assay against CD19+ cancer cells, VECT treated T cells show similar cytotoxicity as the CD19 CAR control cells, indicating VECT does not negatively impact CAR-T cell potency. Conclusion: The VECT microfluidic device can be applied to T cells in various stages of activation, expanding its usefulness to both traditional and rapid manufacturing workflows. Cell mechanics can be used to guide device optimization. The VECT process did not reduce cell proliferation, transduction efficiency, or cytotoxicity. The observation that VECT processed T cells had higher transduction efficiency is interesting to consider as an approach to improve transduction efficiency and reduce vector consumption in next-gen manufuctuing.

823 AAV9-Mediated Overexpression of MBNL Rescues Cardiac Phenotypes in the Microsatellite Expansion Disorder Myotonic Dystrophy Type 1

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Myotonic dystrophy type 1 (DM1) is a multisystemic, autosomal dominant disease caused by a CTG expansion in the 3' untranslated region of the *DMPK* gene. Over half of individuals affected by DM1 have cardiac involvement, such as conduction defects and arrhythmias, which can lead to sudden cardiac death, the second leading cause of death in DM1. RNA containing expanded CUG repeats (CUGexp) transcribed from the expanded *DMPK* allele causes DM1 pathogenesis by disrupting functions of RNA binding proteins, which regulate alternative splicing (AS) transitions during postnatal heart development. For example, muscleblind-like (MBNL) protein family, is sequestered on the CUG_{exp} RNA, resulting in a loss of function. Thereby, misregulation of AS leads to the expression of protein isoforms that are incompatible with adult tissue function, which cause disease features. While many molecular effects of CUG_{exp} RNA have been identified in skeletal muscle, the details of how CUG_{exp} RNA induces cardiac involvement are unknown. We used our DM1 heart mouse model (CUG960), which expresses cardiomyocyte-specific and tetracycline-inducible RNA containing 960 interrupted CUG repeats (CUG960), to determine mechanisms of cardiac pathogenesis by testing for phenotypic rescue. The induced CUG960 mice not only show nuclear RNA foci with MBNL colocalization and splicing defects, but also develop conduction delays and arrhythmia characteristic of DM1 that is reversible upon loss of CUG960 RNA expression. Systemic AAV9 was used for heart-specific overexpression of epitope-tagged MBNL1 and/or MBNL2 that was confirmed in left ventricles and atria of induced CUG960 mice. AAV9-MBNL1, -MBNL2, and MBNL1+2, but not the AAV9-tdTomato control, significantly reduced the disrupted AS events, as well as the prolonged QRS and QTc conduction intervals induced by CUG_{960} RNA. All three cohorts showed rescued heart weight and echo parameters, such as ventricular wall thickness and ejection fraction. Interestingly, AAV9-MBNL1 showed a greater effect in QTc intervals and trends of having better rescue levels in multiple molecular and physiological phenotypes compared to AAV9-MBNL2. Moreover, rescue levels of AAV9-MBNL1 alone were similar to dual expression of AAV9-MBNL1+2. Overall, the data indicate that MBNL1 and MBNL2 both play a crucial role in DM1 cardiac pathogenesis, while MBNL1 may have a greater influence.

824 Characterizing Prototype Fixed Cell-Based Reference Materials for Genomic and Flow Cytometry Measurements

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Lentiviral vectors as a conduit for genetic engineering have emerged as a powerful technology in the field of cell and gene therapies to treat a wide array of diseases including various cancers, autoimmune disorders, and hereditary diseases. As gene and cell therapies are developed, a major limitation is identifying the number of gene insertion replicates due to the indiscriminate lentiviral vector-based genome editing process. Hence, we aim to address that limitation by providing a reference material with known vector insertion copies that has been tested for stability and homogeneity during in-house development and interlaboratory transfer of the materials. Using Jurkat cells with defined vector copy numbers (VCN 0, 1, 2, 3, 4) of GFP-tagged lentiviral vector inserted into the host genome (Paugh et. al., Scientific Reports, 2021), we generated fixed prototype cell-based reference materials to preserve and analyze specific features of the cell, such as nuclear material, fluorescent proteins, surface antigens, and optical scattering properties. We used a mixture of organic solvents to preserve genomic material and present a corresponding validation assay to monitor the stability and interlaboratory transfer of the fixed cells with commonly used viability (DAPI staining) and DNA (qPCR) measurement platforms. Next, MBS crosslinker, in conjunction with microtubule stabilizing buffer, was employed to retain cytosolic fluorescent proteins, cell surface markers, and scattering properties in fixed cells that can serve as a reference for flow cytometry and image-based analysis. Importantly, we show that the fixed prototype reference materials can be fixed in large batches in-house to ensure reproducibility and subsequently distributed between laboratories to allow horizontal comparison of datasets. Taken together, these fixed VCN cells exemplify a high quality, stable, and homogenous reference material that can be applied to assay development and bioprocess validation for gene and cell therapies.

825 Iodine-125 Seeds Implantation Combined with PD-1 Inhibitor Plus Platinum-Based Chemotherapy for Advanced Non-Small Cell Lung Cancer: A Retrospective Study

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Purpose: To evaluate the efficacy and safety of iodine-125 seeds implantation combined with PD-1 inhibitor plus platinum-based chemotherapy for patients with advanced non-small-cell lung cancer (NSCLC). Methods: A total of 21 advanced NSCLC patients were retrospectively analyzed from September 2019 to June 2022, who had undergone iodine-125 seeds implantation combined with

PD-1 inhibitor plus platinum-based chemotherapy. The response of treatment was evaluated according to the evaluation standard for solid tumor efficacy version 1.1, and treatment-related toxicities were evaluated according to NCI-CTCAE version 5.0. The median follow-up time, progression-free survival (PFS) and overall survival (OS) time were calculated by Kaplan Meier method. Log-rank test was used for comparison of survival curves between and among subgroups. The association of dose with response to treatment was analyzed by Pearson's correlation. $p<0.05$ was considered statistically significant. Results: The median follow-up time of these patients was 11.2 months (range: 3.4-23.5 months). The median PFS and OS were 11.2 months (95%CI: 7.4-14.9 months) and 14.3 months (95%CI: 11.0-17.5 months), respectively. Of the 21 patients, 3 (14.3%) experienced a complete response, 5 (23.8%) experienced a partial response, and 13(61.9%) had stable disease. The objective response rate (ORR) was 38.1%, and the disease control rate (DCR) was 100.0%. The most common treatment-related toxicities were radiation pneumonitis (grade 2, 9.5%) and hypothyroidism (grade 1, 9.5%). Treatment-related toxicities of grade 3 or higher were not found. Conclusions: Iodine-125 seeds implantation combined with PD-1 inhibitor plus platinum-based chemotherapy is safe and tolerable, improving the survival of these advanced NSCLC patients. Keywords: NSCLC, Immunotherapy, PD-1, I-125 seeds implantation

Clinical features of the present 21-patient cohort		
Category	N	Percentage (%)
Gender	165	
Male		
Age (yr)		
<65		
≥65		
Performance status (ECOG)		
0-1		
2		
Smoking status		
Smoker		

826 Genomic Characterization of AAV Products Using Multiplex ddPCR and Nanopore Sequencing

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Understanding the identity and integrity of AAV gene therapy products is an essential part in their characterization. Both identity and integrity have the potential to impact therapeutic effect if fragmentation occurs. Understanding this aspect of gene therapy can provide insight into whether the product is intact, fragmented, or potentially being mispackaged. Many approaches to evaluate integrity are available but often introduce bias or provide incomplete information. These methods may be less accurate, provide less information, or misrepresent packaged DNA. Ideally, genomic characterization should include methods to address the 1) ID of the GOI of interest (2) characterization of the amount of full-length gene of interest (GOI) packaged (3) quantification and identification of the mispackaged DNA (4) characterization of the length of DNA (GOI and contaminating) packaged in the AAV vector (5) relationship between GOI and mispackaged DNA. Multiplex digital droplet PCR (ddPCR) has been developed as an effective analytical method to determine the integrity of the product. By measuring the % of connected region for the gene of interest (GOI) with primer sets on opposite ends of the GOI, the percent of intact product can be determined robustly and accurately following DNA amplification. If the two targets on opposite ends of the GOI are positive, then the genome can be considered intact. While Multiplex ddPCR has proven consistent results for % of the full length gene packaged, it does not provide much information on the product identity. Next Gen sequencing, such as Nanopore Sequencing can provide valuable insight into the identity of gene product and other packaged DNA. With Nanopore sequencing, long read sequencing is achieved by reading the packaged DNA strand and aligning it to theoretical reference sequences provided. As the Nanopore sequencing method has the potential to introduce bias, this abstract describes the use of two methods and the value of each approach: Tagmentation Method and the guide RNA Method. In the Tagmentation method, the DNA product is fragmented so adaptors can attach to read the bases. With the guide RNA, DNA sequence is targeted at the ITRS so that a full reading is achieved. As the guide RNA method does not compromise genome integrity through fragmentation, sequencing results show a fragment size distribution that can be used as a comparison to % intact results obtained by Multiplex ddPCR. To obtain information on identity of the product, however the Tagmentation method provides better insight on DNA species identity and information on mispackaging. The combined use of genetic characterization approaches such as multiplex ddPCR, the Next Gen sequencing allows for a more robust and accurate characterization of the packaged DNA length, integrity and identity.

827 CRISPR RNA Targeting Tool Comparison for *UBE3A* Unsilencing in Angelman Syndrome

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Angelman Syndrome (AS) is a rare neurodevelopmental disorder that arises from a dysfunctional or missing maternal *UBE3A* expression in the brain, causing severe intellectual and motor disability. There is currently no cure or effective therapeutic for AS patients. Due to a brain-specific long non-coding RNA transcript known as the UBE3A-antisense (*UBE3A-ATS*), paternal *UBE3A* remains silenced. It is,

therefore, possible to restore brain *UBE3A* expression by reactivating the paternal allele. Recent studies have shown that CRISPR-Cas13 systems can efficiently degrade RNA in many organisms, including mammalian cells (Konermann et al, 2018), and could even be used for CNS disorders (Powell et al., 2022, Morelli et al., 2023). However, more recent studies have detected significant collateral damage of bystander RNA (Tong et al, 2022; Ai et al. 2022). Here, we present for the first time that the recently characterized CRISPR-Csm complex, a multi-protein effector from type III CRISPR systems in prokaryotes (Colognori et al. 2023), provides an even better RNA ablation of *UBE3A-ATS* than Cas13d for Angelman Syndrome. Additionally, we compare it to a new high-fidelity Cas13d (hfCas13d) that was published recently (Tong et al. 2022). To compare the different tools, we used a HEK293T screening system. Plasmids were delivered to the cells to produce the target RNA, consisting of a region of the UBE3A-ATS fused to a green fluorescent protein (GFP). Guides were expressed from plasmids containing the red fluorescent protein mCherry or a blue fluorescent protein BFP, thus giving insight into the delivery of the different RNA targeting tools and collateral damage. Furthermore, we compared the Cas13d and Csm on AS patient iPSC-derived neurons and AS rat primary neurons using lentivirus and the same guides. We delivered the best candidates of hfCas13 and Csm guides to the brain of the AS rat model using an adeno-associated virus (AAV) to measure the *Ube3a and Ube3a-ATS* expression. This study provides the basis for an RNA-targeting CRISPR therapy for the treatment of Angelman Syndrome.

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828 TruStable™: A Fully Integrated Inducible Stable Producer Cell Line for AAV Manufacturing

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Adeno-associated viruses (AAV) are vectors of choice for many gene therapy products. Despite the undeniable suitability of AAV vectors for gene transfer, challenges remain to effectively manufacture these critical medicines for commercial and large patient population applications. Transient transfection is the most common method for the production of recombinant AAV vectors (rAAV). Though versatile, transient transfection suffers from limited scale up potential, as well as supply chain challenges in procurement of GMP grade plasmids. Other approaches involve integration of rAAV transgenes into producer cell lines and a final transduction step with helper viruses to trigger rAAV production. These transduction-based approaches require GMP manufacturing of complex helper viruses and subsequent clearance of these viruses from the final rAAV vector preparation prior to patient dosing. We have therefore developed TruStable, a HEK293-based serum free, suspension adapted cell line that has all components required for rAAV production stably integrated in its genome in order to simplify rAAV production at any manufacturing scale. Core to this system is an inducible, recombination-based molecular production circuit that silences cytotoxic viral elements during initial stages of cell expansion and allows for a simple "on" switch for viral production once an optimum cell culture environment is achieved. Our system uses a two-trigger small molecule switch to turn on rAAV production, providing complete repression of toxic viral elements in the "off" state. In the "on" state, the use of native AAV promoters for replicase and capsid expression ensures a controlled ratio of these viral components for optimum rAAV production. First, a plasmid encoding inducible adenovirus helper genes plus recombinase is stably integrated into the genome of suspension HEK293 cells. This helper cell line is then used as a host cell line to simultaneously integrate recombinase-inducible serotype specific, replicase-capsid plasmid and AAV ITR flanked transgene plasmid to create program specific polyclonal production pools. Single cell cloning of the production pools using an automated single cell isolation system and a high throughput plate based production assay allows us to select monoclonal cell lines that yield high titers with desired critical quality attributes. We have generated several TruStable cell lines that produce self-complementary rAAV vectors expressing a variety of transgenes. These cell lines readily scale into bioreactors and produce consistent titers for at least fifty population doublings demonstrating the scalability and robustness of the system. The combination of molecular production circuits and the ability to select specific clones based on productivity and product quality attributes provides substantial improvement over other rAAV production methodologies. The TruStable producer cell line can be used to package a variety of payloads within a capsid serotype of choice. High titer clones can be obtained in a few weeks to support small to large scale manufacturing runs.

829 AAVone: An All-in-One Plasmid System for Efficient AAV Production

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Recombinant adeno-associated viral (AAV) vector production is seen as complex, with the production scale-up regarded as a major challenge technically, and a large barrier for commercialization. Currently, transient transfection of plasmid into mammalian cells (such as HEK 293) is the strategy most commonly used in clinical grade manufacturing of AAV vectors. Typically, three plasmids are used: one carrying the AAV regulatory (Rep) and structural capsid (Cap) genes, one carrying the ITRs and transgene, and one carrying the specific genes that provide adenoviral helper functions. The transfection approach is fairly rapid and versatile and has been used to produce different serotypes of AAV vectors. However, producing GMP grade of AAV with high consistency, high purity and free of process-related impurities and variants remains a challenge. Production of three plasmids is time-consuming and labor-intensive. To solve those problems, we developed an all-in-one AAV production system, called AAVone (Fig.1). Firstly, we created a mini-pHelper with size down to 8.4 kb by deletion the introns of E2 and E4 expression cassettes. Second, we integrated either AAV helper genes (Rep and Cap) or AAV vector genome into the mini-pHelper and created two version of dual-plasmid systems. Third, we integrated all the necessary elements into one plasmid. Thus, in the AAVone system, Ad helper genes (E2A, E4orf6/7 and VA RNA), AAV helper genes (Rep and Cap), and AAV vector genome are assembled into one plasmid and AAV vectors are simply generated by transfection one plasmid into host cells. The AAVone plasmid has total size of 13.9~17.9kb, depending on the size of AAV vectors. Using AAV2-CMV-EGFP as example, AAVone system achieved unpurified yields of $0.5\sim1.5\times10^{15}$ viral genomes (VG)/L cell culture and average purified yields of $>2\times10^{14}$ VG/L, which is 1.5~3 fold higher than triple-plasmid system. Moreover, AAVone system has low batch variations, as it only needs one plasmid successfully to be transfected into a cell, without the need to co-transfection of the other two plasmids (Fig.2). AAV vectors generated by AAVone and traditional triple-plasmid system has similar infectivity, empty/full ratio, Rep and Cap genomic contaminations, but lower level of rcAAV, AmpR or KanR genomes in both adherent and suspension HEK 293 cells. Moreover, AAVone system uses less plasmid amount, and avoids the ratio optimization steps comparing with the triple-plasmid system. Taken together, AAVone is a simple, economic, but high consistency and high efficiency AAV production system, especially for GMP grade AAV production.

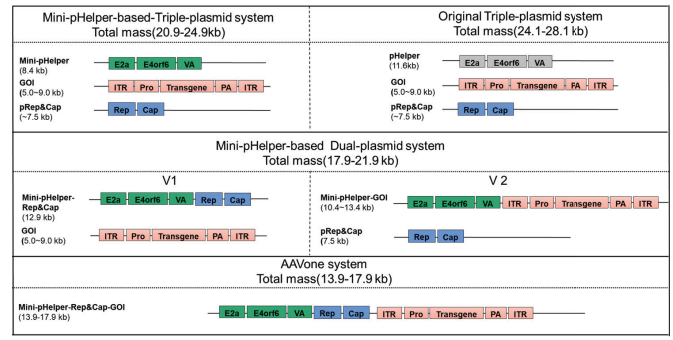

Fig.1 A composite of schematics showing mini-pHelper based triple-plasmid, dual-plasmid and AAVone production system.

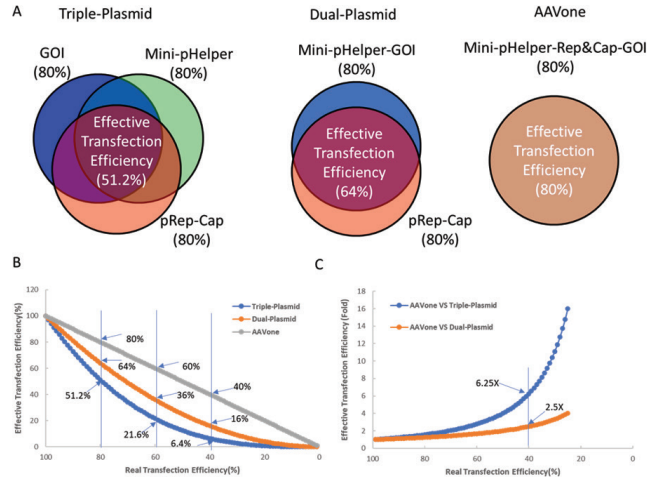

Fig.2 A composite of schematics showing the package efficiency of AAVone system in comparison to triple-plasmid system and dual-plasmid system. AAV package efficiency is depending on the effective transfection efficiency. For the triple-plasmid system, only all three plasmids are successfully transfected into a cell, the cell can produce AAV vector. Thus, the effective transfection efficiency is cube of real transfection efficiency of each plasmid. For the dual-plasmid system, the real transfection efficiency is square of real transfection efficiency. For the AAVone system, the effective transfection efficiency is equal to the real transfection efficiency. For example, if one batch have 80% of real transfection efficiency, the AAV packaging effective transfection efficiency for triple-plasmid, dual-plasmid and AAVone are 51.4%, 64% and 80%, respectively(A). AAV package efficiency would dramatically decrease when the batch has low real transfection efficiency in triple-plasmid system, but only slight decrease in AAVone system (B). At 40% real transfection condition, the effective transfection efficiency of AAVone system is 6.25 times of triple-plasmid system(C). Taken together, AAVone is a high consistency and high efficiency AAV production system.

830 *In Vivo* Gene Transfer of Doggybone DNA Carrying High-Expressing Factor IX Expression Cassettes

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Doggybone™ DNA (dbDNA™) is a linear, double stranded, and covalently closed synthetic DNA construct. It eliminates the bacterial sequence and shows high expression profile. We explored the potential of adopting dbDNA in nonviral gene transfer of human factor IX (hFIX) for treatment of hemophilia B. The dbDNA and plasmid vector containing a high-expressing hFIX cassette driven by a hepatic control region (HCR) and hepatocyte specific human α-1 antitrypsin

(hAAT) promoter were administered into a murine model to test their expressing capability, respectively. First, to determine the optimal dosage, an initial test was performed by hydrodynamically injecting 5, 10, 20, and 50 µg of dbDNA and plasmid DNA to C57BL6J mice. Mouse plasma samples were collected on day 3, 10 and every 10 days afterwards and hFIX expression levels were evaluated by ELISA. The results showed a clear dose response in plasmid DNA treated group, whereas the mice treated with 20 µg dbDNA had more consistently high expression levels among different dosages throughout the 8 weeks of monitoring period. Thus, we proceeded with 20 µg as the dose of injection in subsequent studies. Next, we screened for the best candidate of hFIX expression cassettes for the dbDNA expression with least toxicity. Mice were hydrodynamically injected with 20 µg of two control plasmid DNAs and five dbDNAs containing various hFIX expression cassettes carrying hFIX cDNA combined with various arrangements of Intron A and 3'UTR. Similarly, the plasma samples were collected on day 1, 3, and every 10 days afterwards. With the same hFIX expression cassettes, dbDNA showed higher expression level of hFIX (1.5-2 folds) compared to plasmid DNA and sustained until the end of the study. With comparison of different expression cassettes, hFIX cDNA combined with Intron A in its natural position or incorporated right after the promoter with an additional 3'-UTR achieved higher expression levels. ALT levels in plasma of all dbDNA and plasmid DNA treated mice transiently elevated initially after hydrodynamic delivery and decreased to a normal range after day 3 of injection until the final collection date. Mouse livers were collected 7 weeks after injection. No significant liver damage was observed in dbDNA or plasmid DNA injected mice by histology analysis of the liver sections. We continue to modify the hFIX expression cassettes in dbDNA by adding various sequences to further enhance the expression levels. Durability and dose ranging will also be monitored to test dbDNA as a long-term, high expressing vector for in vivo gene transfer. These results indicate that dbDNA is potentially a good candidate for nonviral gene transfer.

831 Scalable Expansion Culture of Human Primary T Cells

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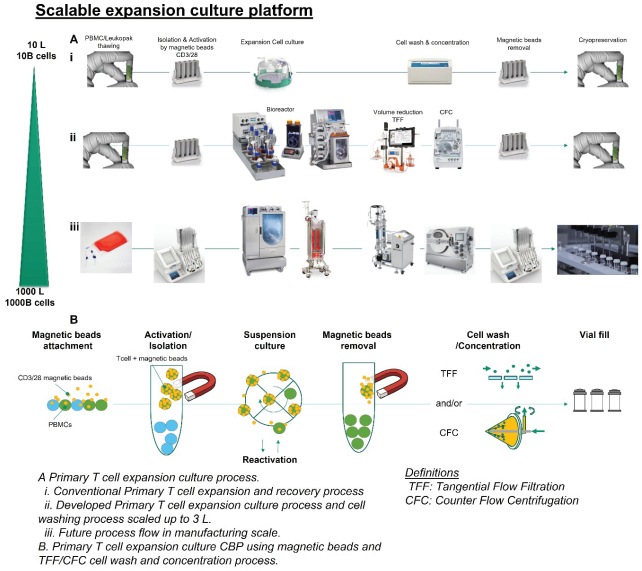
Autologous T cell-based therapies have rapidly become some of the most promising treatments for blood and other cancers. Despite their clinical success, several obstacles related to the time, cost, and quality of starting from the patients' own T cells have prevented wider accessibility to these life-saving treatments. By contrast, using T cells from healthy donors can potentially provide large amounts of highly functional cells that are less expensive to manufacture and are available "off-the-shelf". To transition allogeneic T cell therapies from research to commercial manufacturing, a scalable and reproducible cell expansion process is important. Moreover, for clinical application, it is advantageous to use a single use, closed and controlled environment. At FUJIFILM Cellular Dynamics, Inc. (FCDI), we have experience in process optimization with various culture formats. We created a closed and scalable 3D culture process for primary T cell expansion and reactivation method using magnetic beads and bioreactors. Under optimized conditions with 3L bioreactor, we achieved over 2000-fold increase after 14 days while maintaining comparable cell quality including immune cell marker expression and viability. In addition, closed cell washing and concentration by counterflow centrifugation was demonstrated with high viability and a recovery ratio over 80%. We also showed assessments using a small-scale bioreactor to show process window of primary T cells for process intensification. Together, these established Primary T cell scale-up and processing methods allow for the routine manufacture of over 1000 billion Primary T cells per batch.



A Primary T cell expansion culture process.
i. Conventional Primary T cell expansion and recovery process
ii. Developed Primary T cell expansion culture process and cell washing process scaled up to 3 L.
iii. Future process flow in manufacturing scale.
B. Primary T cell expansion culture CBP using magnetic beads and TFF/CFC cell wash and concentration process.

832 Engineered AAV9 Variants for Efficient Muscle Transduction

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Systematic administration of Adeno-associated virus (AAV) vectors expressing functional genes represents a promising treatment for muscular dystrophy. However, very high dose of AAV is required due to its low transduction efficiency in the muscle after intravenous delivery. We aim to engineer novel AAV capsids with enhanced transduction efficiency in muscles using our AAVarta platform, an AI-aided AAV capsid evolution discovery technology. Based on AAV9 capsid, random peptide insertion AAV capsid libraries were generated and screened in both mice and non-human primates (NHP). The screening process relies on muscle-specific viral mRNA recovery by using an in-house developed potent synthetic muscle promoter SCC45. After two rounds of screening in 3 mouse strains (C57BL/6, BALB/c and DBA/2j-mdx) and one round in NHP, the top capsid candidates were selected for individual validation. By using GFP and firefly luciferase reporter genes, several variants show dramatic improvements (up to 113 folds) in the mouse skeletal muscles compared to AAV9. One variant AVT913 shows 26-fold enhanced transduction at mRNA level in the heart.

Of note, variant AVT919 shows significant improvements in triceps and tibialis anterior over MyoAAV2A, a benchmark myotropic AAV capsid. Although the majority of top variants show improved muscle transduction and reduced liver targeting, one particular variant AVT908 demonstrates enhanced efficiency in both muscle and liver. To confirm if similar tissue-tropic patterns can be recapitulated in NHP, those variants together with additional candidates were individually packaged and intravenously injected to adult cynomolgus macaques. The characteristic results would reveal the cross-species translatability. We believe that those engineered myotropic AAV capsids would provide efficient delivery platforms for muscle-targeting gene therapy.

833 Selective Ablation of Solid Tumors with Dysfunctional p53 Signaling Using a Suicide Gene Delivered via Fusogenix Proteolipid Vehicles

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While chemotherapy is a key treatment strategy for many solid tumors, it is rarely curative as patients will eventually become resistant. In this study, we sought to develop an effective suicide gene therapy approach for solid tumors that specifically exploits the unique transcriptional activation state of the tumor suppressor, p53. We have developed a Fusogenix proteolipid vehicle (PLV) formulation to deliver a plasmid DNA vector where the p53 promoter is included upstream of the inducible suicide gene, inducible caspase-9 (iCasp9). The addition of the small molecule dimerizer agent, rapamycin (RAPA), facilitates cleavage and activation of iCasp9 leading to caspase-mediated apoptosis in cells with elevated p53 promoter activity. We observed a high degree of apoptotic cell death, as measured by live-cell imaging, as well as Annexin V and TUNEL flow cytometry. No cell death was observed in cells with intact p53 such as the fibroblast cell line IMR-90. Systemic delivery of the p53-iCasp9 vector to mice bearing subcutaneous H1299 tumors results in robust apoptosis induction upon activation with RAPA that effectively controls tumor growth over time. When p53-iCasp9 was combined with the anti-CTLA-4 (α-CTLA4) checkpoint inhibitor, treatment was able to reduce the total metastatic burden in the poorly immunogenic B16F10 melanoma lung metastasis model. Treatment correlated with an increase in activated CD4 T lymphocytes and a reduction in suppressive CD4 T_{reg} cells - leading to improved recognition and elimination of B16F10 cells *ex vivo*. In conclusion, we describe a PLV based suicide gene therapy approach for the treatment of cancer with high selectivity for tumors with dysregulated p53 transcriptional activation.

834 Therapeutic Efficacy of Retroviral Replicating Vector Toca 511 (vocimagene amiretrorepvec) -Mediated Prodrug Activator Gene Therapy for Lung Cancer

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Despite the development in recent years of targeted therapy and immune checkpoint inhibitor therapy, lung cancer (LC) remains one of the most lethal cancers, thus new therapeutic strategies are urgently needed. Retroviral replicating vectors (RRV) are capable of highly efficient replication and transduction in cancer cells. Prodrug activator gene therapy with Toca 511 (*vocimagene amiretrorepvec*), an RRV encoding yeast cytosine deaminase (yCD), which converts the prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU) within Toca 511-infected cells, has been shown to achieve significantly enhanced survival benefit in a variety of preclinical tumor models. In an international randomized Phase 2/3 clinical trial for patients with recurrent high-grade glioma, Toca 511/5-FC treatment did not meet its endpoints overall, but demonstrated highly promising evidence of survival benefit in pre-specified patient subgroups. Therefore, we evaluated the therapeutic activity of RRV-mediated prodrug activator gene therapy in LC-derived cell lines and in a preclinical model for metastatic LC.We first quantitated the replication kinetics of RRV in vitro in human (A549, H226, SBC-3) and murine (Ex-3LL) LC cell lines. The percentage of GFP-positive cells were monitored by flow cytometry every 2-3 days after inoculation with RRV expressing GFP as a reporter gene (RRV-GFP) at a multiplicity of infection (MOI) of 0.01. In both human and mouse LC lines, RRV-GFP showed rapid viral replication resulting in high levels of transduction, as indicated by the percentages of GFP-positive cells reaching nearly 90% over time.Next, we evaluated in vitro cytotoxicity by MTS assay after exposure to a series of 5-FC concentrations in LC cells transduced with Toca 511. Compared to controls, Toca 511-transduced A549 and Ex-3LL LC cells showed significant growth inhibition (>80%) after exposure to 1.0 mM and 0.01 mM 5-FC, respectively. Finally, we evaluated the in vivo efficacy of Toca 511/5-FC treatment in subcutaneous tumor models of A549 in immunodeficient athymic nude mice and Ex-3LL in immunocompetent syngeneic mice. In both models, Toca 511-transduced tumors treated with 5-FC showed highly significant growth inhibition compared to control groups ($p<0.0001$). Especially in the Ex-3LL tumor model, 4 out of 5 mice showed complete tumor regression. Furthermore, we established orthotopic pleural dissemination models using luciferase-marked A549 and Ex-3LL tumors to monitor tumor burden by optical imaging of tumor bioluminescence. As compared with their respective control groups, the Toca 511/5-FC treatment group showed significant reductions in mean signal intensity in both A549 ($p=0.0147$) and Ex-3LL ($p=0.0326$) models. Thus, RRV can serve as highly efficient vehicles

for gene delivery to LC, and these in vitro and in vivo data indicated the translational potential of RRV-mediated prodrug activator gene therapy with Toca 511/5-FC as a novel therapeutic strategy for pulmonary malignancies.

835 Modification of the AAV Serotype rh.10 Capsid to Enhance CNS Gene Transfer

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Nonhuman primate serotype AAVrh.10 has been demonstrated to effectively transfer genes to the CNS following direct administration (Sondhi D et al, Sci Trans Med 2020; 12: eabb5413) or via cerebrospinal fluid (Rosenberg JB et al, Hum Gene Ther Clin Dev 2018; 29:24). We hypothesized that, for a given dose, CNS distribution of AAVrh.10 could be further improved by genetic modification of capsid loop IV with sequences designed to broaden distribution. Following initial screening of multiple variants, the AAVrh.10S2 capsid was selected, where amino acids 452-459 in loop IV were replaced with DGAATKQ, a sequence modified from AAV9 CAP-B10 (Goertsen D et al, Nat Neurosci 2022; 25:106). AAVrh.10S2 coding for mCherry was assessed *in vitro* in cell lines U87 (glioma), SVGp12 (glial) and SH-SY5Y (neuroblastoma) and *in vivo* following direct intrahippocampal administration of AAVrh.10S2mCherry to C57Bl/6 mice. In all neural cell lines, expression of mCherry was significantly greater (average 5.8-fold all cell lines) for AAVrh.10S2mCherry than for the same dose (10^4 gc/cell) of the wildtype AAVrh.10mCherry control ($p<0.05$, all cell types). CNS expression of mCherry was assessed 4 weeks following intrahippocampal administration of $2.5x10^{10}$ gc of AAVrh.10S2mCherry compared to AAVrh.10mCherry. With the same dose, mCherry mRNA levels in the hippocampus and cortex were higher with AAVrh.10S2 mCherry compared to AAVrh.10mCherry (hippocampus 55-fold, cortex 7.6-fold; $p<0.05$ by ANOVA). Consistent with the mRNA data, protein expression with AAVrh.10mCherry in the hippocampus was 17.7± 2.6 ng/μg protein and 5.9± 0.5 ng/μg in the cortex. In contrast, with the same dose, hippocampus mCherry levels with AAVrh.10S2mCherry were 39.8 ± 1.8 ng/μg and in the cortex were 17.7± 2.2 ng/μg, >2-fold higher than with the unmodified AAVrh.10 vector ($p<0.001$ by ANOVA). In summary, the modified AAVrh.10S2 capsid has enhanced CNS distribution *in vivo*, providing greater brain transduction compared to unmodified AAVrh.10 capsid following direct intraparenchymal administration. Pending assessment in larger species, the AAVrh.10S2 capsid may be useful for treating diffuse neurological diseases.

837 Ultra-Pure Single Molecule Guide RNAs for Therapeutic CRISPR Gene Editing

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The clustered regularly interspaced short palindromic repeats (CRISPR) gene editing has an intriguing potential to cure many diseases of unmet needs. A CRISPR gene editor is composed of Cas protein(s) and guide RNA(s), and the spacer sequence in the guide RNA (gRNA) determines where the editor introduces genetic changes. Therefore, it is crucial that these gRNAs have minimum impurities such as truncated byproducts, including n+1, n-1, n-2, and n-3 in particular, all of which bind to Cas proteins leading to un-intended genetic modifications. High-efficiency off-target editing is well-documented in the literature. It was reported that chemical modifications improved the on-target editing efficiency while decreasing the off-target editing. Guide RNAs can be produced either in cells from delivered plasmids or by in vitro transcription, both of which do not allow chemical modifications. Chemical synthesis provides unique access to modified gRNAs. Nowadays, automated solid phase synthesis of oligonucleotides of ~20 nt in length works at kilogram scales; however, it is still a daunting challenge for long RNA molecules because of their 2'-OH groups. With the recent new development of 2'-protecting groups, 25~35% crude yields for syntheses of 100mer sgRNAs were reported, which was equivalent to 98.6~98.9% stepwise coupling efficiency. Obviously, to deliver high-grade sgRNAs, we must overcome two major barriers, namely low overall yields and extremely difficult purification. To evaluate the efficacy of truncated guide RNAs, we have synthesized 92mer (n+1), 90mer (n-1), 89mer (n-2), and 88mer (n-3) of a chemically ligated guide RNA (l2gRNA, 91mer) targeting HBV genome. The ligation groups were introduced at the final coupling step (5'-end), and thus most of the truncated segments were not reactive and remained after ligation. The product composing a single spacer sequence was ensured by careful polyacrylamide gel electrophoresis (PAGE) separations of the crRNA segment (32 nt) and optional PAGE separations of tracrRNA segments (36 nt and 23 nt), followed by chemical ligations and one additional PAGE purification for the final products. These l2gRNAs were then mixed on purpose and submitted for LC-MS analysis. Our data indicated, in comparison to good resolutions of short RNA segments, these products gave one overlapped sharp peak in HPLC trace as reported in the literature, and that because of many charged states of molecular ions and adducts, it is very challenging to develop a universal standard analytical method to quantify the product purities. In addition, the truncated lgRNAs showed only slightly decreased or similar efficacy (80~91%) as the full-length l2gRNA (95%) in AD38 Cas9 stable cell line. Based on these data, we caution that chemically synthesized long guide RNAs, in particular, sgRNAs (~100 nt) and pegRNAs (>120 nt), contain assemblies of inseparable truncated byproducts of various spacer sequences, which can give rise to unpredictable off-target editing. LgRNA technology provides scalable and efficient access to ultra-pure long guide RNAs, which are critical for clinical applications.

838 Interleukin-15 and Interleukin-2 Promote Persistence of FcεRIγ Negative (g-NK) Natural Killer Cells in Mice

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Purpose: Natural killer (NK) cells have intrinsic abilities to identify and eliminate tumor cells and virally infected cells. Enhancing the potency of therapeutic NK cells and improve their in vivo persistence are critical for the success of NK cell immunotherapy. A subset of human NK cells lacking expression of FcεRIγ (g-NK cells) is known to have a multifold increase in antibody-dependent effector functions after CD16 crosslinking. We sought to evaluate the in vivo persistence of g-NK cells with interleukin-15 (IL-15) and interleukin-2 (IL-2) treatment in mice. **Methods:** g-NK cells ($CD45^+7\text{-}AAD^-CD3^-CD56^+Fc\varepsilon RI\gamma^-$ lymphocytes) were expanded from cryopreserved PBMCs using a published method. A single dose of 1.0×10^7 expanded g-NK (freshly expanded or cryopreserved) or conventional NK cells (cNK, cryopreserved) was injected intravenously through the tail vein into 6-8 weeks old female NSG mice. Mice immediately received either recombinant human IL-15 (2 μg/mouse intraperitoneally every 3 days, n=9 mice) or human IL-2 (10 μg/mouse intraperitoneally every 2 days, n=6 mice). Peripheral blood (50 μL) was drawn at post-cell injection days 6, 16, 26, and 31 for IL-15 treated mice and days 3, 6, 12, 19, 26, and 33 for IL-2 treated mice for immediate flow cytometric analysis of g-NK cells. Mice were euthanized on day 31 and 33 for IL-15 and IL-2 treatment, respectively. Mice body weights were monitored every other day throughout the study. **Results:** In the IL-15 treated mice, persistence of cryopreserved g-NK cells was > 10 times higher than that seen with cryopreserved cNK cells in peripheral blood at days 6, 16, 26, and 31 post cell injection ($P < 0.001$). Persistence of g-NK cells were also higher than that of cNK cells in spleen and bone marrow when the mice were euthanized at day 31 ($P < 0.001$). Cyropreserved g-NK cells persisted at levels comparable to that in fresh g-NK cells for at least 26 days post cell transfer. In the IL-2 treated mice, after single dose of cryopreserved g-NK injection, the blood g-NK cell numbers increased significantly and peaked at day 12 post cell injection, then dropped slowly from day 19-33. Both IL-15 and IL-2 treatments have minimal impact on the body weight of mice compared to untreated control mice within the study period indicating minimal systemic toxicity. **Conclusion:** Both IL-15 and IL-2 are effective in enhancing in vivo persistence of g-NK cells with minimal systemic toxicities. Cryopreserved g-NK warrants further evaluation as an off-the-shelf cell immunotherapy.

840 Anti-AAV1 Antibodies Do Not Inhibit Neuronal Transduction after Direct Brain Administration with AAV1

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Adeno-associated viruses (AAVs) are the leading vectors of choice for gene therapy. One limitation of these vectors is that recombinant AAV vectors are derived from their wild-type counterpart, which can naturally infect humans and give rise to anti-AAV antibodies. These anti-AAV antibodies adversely impact gene therapy outcomes because they inhibit transduction efficiency, and preclinical research animals and human patients must be carefully screened for anti-AAV serum titers. Whether anti-AAV serum titers are relevant in immune-privileged locations such as the brain is still an open question. To address this question, we developed an experimental strategy to evaluate the impact of anti-AAV1 antibodies on neuronal transduction in the Long Evans (LE) rat brain. Specifically, we injected LE rats intramuscularly with either empty AAV1 capsids or PBS as a control. Two weeks later, we used blood serum samples to confirm the presence of anti-AAV1 antibodies, and a strong anti-AAV1 response was observed in rats that received the empty AAV1 capsid injections. We then delivered AAV1-CAG-eGFP (1e10 GC delivered in 2 μL) unilaterally directly into the substantia nigra (SN) and analyzed GFP expression at 4 months post-injection by immunofluorescence. There was no difference in GFP expression in the SN between the two groups of rats suggesting that the presence of anti-AAV1 antibodies in the periphery does not decrease neuronal transduction after direct brain delivery. This knowledge can inform us about patient inclusion in clinical trials for AAV-based gene therapy for neurological disorders when the vector is delivered directly into the brain. Though we also found that AAV1 injection into the brain results in the production of anti-AAV titers in serum, we would not expect this Anti-Drug-Antibody (ADA) induction to limit future CNS AAV re-dosing.

841 Efficient Mini dCas13-Mediated Base Editing for Personalized Treatment of Duchenne Muscular Dystrophy

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Background: Approximately 10% of monogenic diseases are caused by nonsense point mutations that generate premature termination codons (PTCs), leading to a truncated protein and nonsense-mediated decay of mutant mRNAs. Specifically, ~50% of these point mutations can be reversed by A to G conversion with adenine base editors (ABE) to restore protein expression. Here, we demonstrate a mini-dCas13X-mediated RNA adenine base editing (mxABE) strategy to treat nonsense mutation-related monogenic diseases via A-to-G editing in a genetically humanized mouse model of Duchenne muscular dystrophy (DMD). **Methods**: We identified a nonsense point mutation (c.4174C>T, p.Gln1392*) that introduced a PTC in the *DMD* gene exon 30 of a patient and validated its pathogenicity in a genetically humanized DMD murine model ($DMD^{Ex30mut}$). A single adeno-associated virus (AAV) containing mxABE was delivered to $DMD^{Ex30mut}$ mice to correct the PTC mutation in the *DMD* gene. To further demonstrate the efficacy and safety of the single AAV-packaged

mxABE strategy in large mammals, two gRNAs flanking porcine *DMD* gene exon30 were designed, and porcine fetal fibroblasts (PFFs) isolated from a 30-day-old fetus of Duroc pig were transfected with donor template and CRISPR plasmids. Forty-eight cell lines that exhibited good morphology and viability were carefully singled out, and two knock-in cell lines were pooled and used as nuclear donors to produce the DMD porcine models. **Results**: In this humanized DMD^{E30mut} mouse model, a single AAV-packaged mxABE reached A-to-G editing rates up to 84% *in vivo*, much higher (at least 20 folds) than that reported in previous studies using other RNA editing modalities (Fig. 1A,B). Furthermore, mxABE restored robust expression of dystrophin protein to over 50% of wildtype level by PTC read-through in multiple muscle tissues. Importantly, systemic delivery of mxABE by AAV can also rescue dystrophin expression (37% in diaphragm, 6% in tibialis anterior and 54% in heart muscle on average, compared to wildtype level) (Fig. 1C), serum creatinine kinase levels and muscle function and muscle function (Fig. 1D, E and F). **Conclusions**: These data suggested mxABE may overcome the current limitations of the CRISPR system for the treatments of DMD and other monogenic diseases. Continued development of this mxABE approach for DMD in large animals may provide an essential step toward the clinical translation of alternative gene-editing technology in the near future.

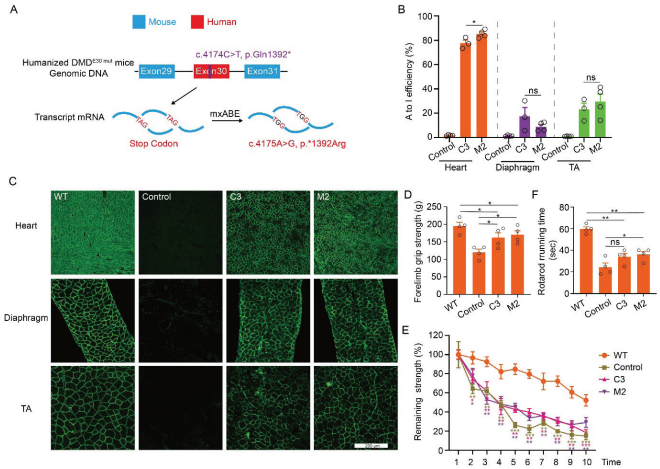

Figure 1. Systemic delivery of AAV-mxABE rescues dystrophin expression and muscle function in multiple organs.
A, Schematic diagram of nucleotide editing strategy in humanized DMD mice. The mutation exon30 generated a premature stop codon, and can be corrected by mxABE system. B, Measurement by deep sequencing of dystrophin transcripts of the targeted A to I editing efficiency in TA, Diaphragm , and heart (n=4). C, Immunohistochemistry analysis shows restoration of dystrophin expression in the TA, Diaphragm, and heart of DMD$^{E30 mut}$ mice 6 weeks after injection. Dystrophin was shown in green. Scale bar, 200 μm. Forelimb grip strength (D), Rotarod (E) and remaining strength (F) were measured in WT, DMD^{E30mut}mice, and DMD$^{E30 mut}$ mice treated with AAV particles (n=6). Data shown as mean ± SEM. Different asterisks represent statistical significance (P<0.01) in multiple comparison test using ANOVA.

842 *In Utero* Lipid Nanoparticle Targeting of the Fetal Skin for mRNA Therapies

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Introduction: The rationale for therapeutic in utero gene editing is to harness normal developmental ontogeny to institute therapy in a more efficient and safe manner prior to the onset of pathology. Among the properties of the fetus that make it attractive for *in vivo* gene editing is the abundance and accessibility of progenitor cells, including stem cells of organs that are difficult to target after birth. The skin exemplifies this premise with genetic skin disorders, such as epidermolysis bullosa, often leading to diffuse blistering shortly after birth. Critical to harnessing the potential of new gene editing and mRNA therapies for such diseases is the ability to diffusely and efficiently target skin epithelial progenitor cells. In the current study we assess the ability of lipid nanoparticles (LNPs) delivered to the amniotic cavity in a fluorescent reporter mouse model to target the fetal skin. We hypothesize that fetal skin has an increased amount of stem cells relative to neonatal skin and that fetal skin stem cells can be readily targeted by intra-amniotic (IA) delivery of LNPs in a gestational age dependent manner. **Methods:** We performed IA injection of LNPs containing Cre recombinase mRNA (LNP-Cre) into mTmG heterozygous fetuses at gestational day (E) 12 (n=10) and E16 (n=10), as well as control IA PBS injections at E12 (n=10). mTmG mice express GFP in all cell types following Cre mediated excision of the mT gene. After birth, mice were harvested at postnatal day (P) 1, 7, and 30 with skin assessed for GFP expression by fluorescent stereomicroscopy and flow cytometry. Additionally, skin of uninjected mTmG heterozygous mice was harvested at E14 and P1 and the relative abundance of CD34+ bulge stem cells was quantified by flow cytometry. **Results:** Mice injected at E12 and imaged at P1 demonstrated a stippled pattern of GFP expression on the scalp and extremities that extended linearly down the abdomen and back with scattered areas of hyperintensity. By P7 these mice demonstrated diffuse GFP expression across the entire body that persisted at P30. In contrast, experimental mice injected at E16 and control mice demonstrated no editing. For injected E12 mice, flow cytometry at P7 demonstrated an average of 5% editing of CD34+ bulge skin stem cells, while control and E16 injected mice had no editing. Additionally, flow cytometry of skin harvested from uninjected mice at E14 demonstrated a greater proportion of CD34+ stem cells compared to skin harvested at P1 (7.66 vs 0.86%, p=0.0003). **Conclusion:** Mouse fetal skin, including bulge skin stem cells, is accessible for mRNA delivery via intra-amniotic LNP injection. The efficiency of skin stem cell targeting is highly dependent on the gestational age at which the LNP is injected, with increased efficiency earlier in gestation likely reflecting the development of a protective keratinized skin layer later in gestation. These studies suggest a possible LNP approach to deliver therapeutics to the skin for genetic diseases.

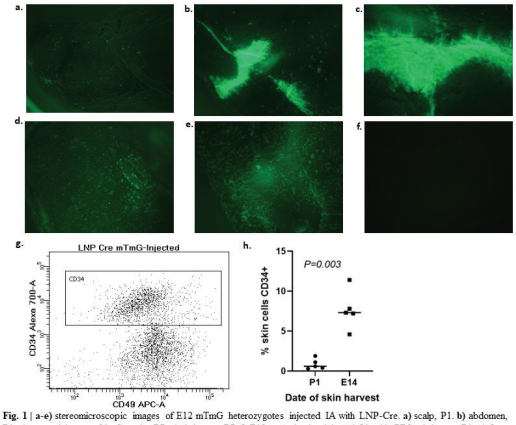

Fig. 1 | a-e) stereomicroscopic images of E12 mTmG heterozygotes injected IA with LNP-Cre. **a)** scalp, P1. **b)** abdomen, P1. **c)** zoomed image of b. **d)** scalp, P7. **e)** abdomen, P7. **f)** E12 control mice injected IA with PBS; abdomen, P1. **g)** flow cytometry of E14 skin sample staining for CD49 alpha-6 integrin and CD34+ cells. Box drawn around CD34+ bulge stem cells. **h)** % skin cells staining positive for CD34+ at E14 vs P1 for uninjected mice.

843 A Cell Subtype-Mediated Mechanism Informs Cell Therapy Strategy for Discogenic Low Back Pain

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Introduction: Low back pain (LBP) is a leading cause of disability affecting almost 80% of the adult population, and 40% of LBP is attributed to intervertebral disc (IVD) degeneration. However, radiographic signs of IVD degeneration do not always correlate with painful discs. The mechanisms that determine which degenerated discs drive the pain are yet to be fully revealed. Loss of nucleus pulposus cells (NPCs) correlates with disc degeneration. We hypothesize that NPC affected by specific environmental stressors trigger the onset of low back pain. **Methods**: Degenerated IVD tissue freshly harvested from surgical discards or human cadavers with approved protocols were compared with single-cell RNA-seq (scRNA-seq), including 3 **back pain-inducing** IVDs (**bpIVD**) and 4 **asymptomatic** IVDs (**aIVD)** from different individuals. Further subset NPCs from bpIVD (**bpNPC**) or aIVD (**aNPC**) for further analyses. Replated aNPCs in 2D tissue culture and transformed them from non-stressed status (**nsNPC**) into a stressed status (**sNPC**) by applying pro-inflammatory cytokine IL-1β, low pH, and low glucose. Nociception-activating effects of nNPC and nsNPC in were determined in vitro in a co-culture with iPSC-derived nociceptors (**iNOC**) using microfluidic devices. The sNPC, nsNPC, and saline were injected into healthy rat IVDs followed by μMRI and biobehavioral tests for up to 8 weeks. Post-sacrifice, IVDs were harvested and evaluated for nociceptive markers expression and histology IHC. For statistics, 3-way ANOVA and a critical significance level of 5% was used. **Results:** ScRNA-seq identified NPCs as the major cell type affected by LBP condition (Fig. 1A). Some clusters, particularly NPC1^{MMP3+}, were found associated with bpIVDs because 62% cells of this subtype were derived from bpIVDs (Fig. 1A). A pseudo-time trajectory analysis shows the trend from aIVD-associated NPC7 to aIVD-associated NPC6, and a bpIVD-associated NPC1 side branch in the middle (Fig. 1B). Within NPC1 subtype, the cells solely derived from bpIVD were annotated as **bpNPC1** and further examined along with NPC6 and NPC7 cells derived from aIVD (Fig. 1C). The **bpNPC1** subset showed the upregulated nociception and neuron outgrowth regulated by NGF. These data suggest that NPC1 cluster might be the pain-inducing cell subtype. We recreated the NPC1 subtype with combinatory stimulation of NPCs *in vitro* with cell stressors (Fig. 1D) resulted in an upregulation of TNF-α, IL-1β, IL8, MMP3, and NGF (Fig. 1E). sNPC showed bpNPC1-like transcriptomes as evidenced by 1) more overlap of expressed genes with bpNPC1 than bpNPC (Fig. 1F) and 2) more bpNPC1-like MMP3 expressions than nsNPC (Fig. 1G). The sNPC induced functional response from iNOC but nsNPC did not (Fig. H- N). In the animal study, the intradiscal injection (Fig. 1O) led to expression of nociceptive markers TRPV1, Gap43 and CGRP only in sNPC-injected group (Fig, 1P) and increased nociceptive behavior compared to nsNPCs or saline-injected groups (Fig. 1Q). **Conclusion**: This study discovered that a subtype of NPC generated under stress are responsible for discogenic low back pain. This NPC-mediated pain mechanism will inform future NPC-focused clinical therapies that are more approachable than regenerating the entire IVDs.

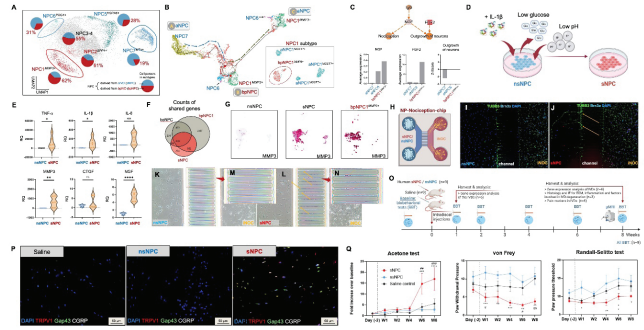

*Fig. 1. (A) NPC cell atlas in IVDs. Pie charts show cell percent derived from different IVDs. (B) Pseudo-time trajectory shows the developmental direction among subtypes. Terminal A, B, and C are dominated by aNPC, bpNPC, and aNPC respectively. (C) NGF-regulated network for bpNPC1 predicted with Qiagen IPA. Combinatory stressors (D) recreated stress phenotype expressing stress markers (E). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns p>0.05. (F) Counts of expressed genes shared by two samples. (G) NPC cell atlas colored with red representing expression level of NPC1 marker MMP3. In a microfluidic NP-nociception-chip (H), only nNPC recruited iNOC axon outgrowth as evidenced by TUBB3 expression (I) and (J), and optical microscope images showing the axons outgrowth in the middle channels (K), (L), (M), and (N). (O) Experimental design of animal study testing sNPC-injected group compared to nsNPC-injected or saline-injected groups. (P) IVD staining for DAPI and pain markers TRPV1, Gap43, and CGRP in IVDs. Scale bar = 50 μm. (Q) Biobehavioral tests (BBTs) showed pain-related outcomes in Acetone test, von Frey test, and Randall-Selitto test at 2 days prior to injections, week 1, 2, 4, 6, and 8 post injections. (n=9) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (compared to saline control), #p<0.05, ##p<0.01 (compared to nsNPC).*

844 Ministring DNA Encoding Human Factor VIII Gene: A Novel Paradigm of Nonviral Gene Therapy for Hemophilia A Patients

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Ministring DNA (msDNA) is a novel double-stranded linear DNA minivector with covalently closed ends. The DNA minivector eliminates bacterial plasmid sequences and any CpG motifs, conferring a safe, redosable, and effective non-viral gene delivery profile. Hemophilia A is a genetic disorder caused by missing or defective coagulation factor VIII (FVIII gene) protein, whereby gene therapy represents a promising treatment approach to correct this disorder. However, there is unmet need for a controlled and titratable gene therapeutic for HemA patients. We evaluated and optimized the FVIII construct (HP-hFVIII) that was integrated into the msDNA platform to achieve the advantages of a safe and redosable genetic medicine. The msDNA encoding human FVIII (hFVIII) gene with a hepatocyte specific promotor (msDNA-HP-hFVIII) was generated (8.1kb) from the plasmid pHP-hFVIII (10kb). An msDNA construct, msDNA-CAG-W-hFVIII driven by the ubiquitous, highly efficient promoter CAG (7.2 kb) served as the vector control. We evaluated the impact of genetic regulatory sequences in a dose-dependent manner. Groups of immunocompetent murine FVIII knock out mice (Hem A mice) had 1 μg (0.04 mg/kg) of respective msDNAs and the parent plasmid, pHP-hFVIII, administered via hydrodynamic injection. The plasma of the treated mice was collected on days 1, 7, 14 and each subsequent 14-day period to examine FVIII activity in treated mice via activated Partial Thromboplastin Time (aPTT) tests. On day 1 post-vector administration, the Hem A mice treated with 1 μg msDNA-HP-hFVIII produced more than 4,000% wild type level of FVIII activity wild type levels in human plasma defined as 100%). Relative to plasmid, msDNA generated 4-fold higher FVIII. In

addition, the mice treated msDNA-CAG-W-hFVIII exhibited 14,000% FVIII activity. However, the high levels of human FVIII expression rapidly induced high levels of anti-hFVIII inhibitor titers, resulting in elimination of FVIII expression to undetectable levels in less than one month. To avoid inhibitor formation, we reduced the DNA dose from 1 μg to either 0.2 μg or 0.04 μg (0.008 or 0.0016 mg/kg, respectively). In the 0.2 μg group, only msDNA-CAG-W-hFVIII treated mice developed inhibitors, whereas both msDNA-L6-hFVIII and pHP-hFVIII treated group had persistent expression levels around 300% and 100%, respectively without antibody formation for over 6 months (experimental duration). Similar trends were observed in the 0.04 μg groups. In particular, mice treated with 0.04 μg msDNA-HP-hFVIII produced sustained levels of FVIII activity (around 100%) more than 6 months post-single systemic administration. In all cases, msDNA showed significantly higher FVIII levels compared to the plasmid DNA (>3-fold). The results revealed a linear dose-dependent response and the durability of FVIII expression in the msDNA-HP-hFVIII treated mice following a single treatment. In conclusion, a single hydrodynamic delivery of the hepatocyte specific msDNA-HP-hFVIII in Hem A mice was highly efficient and the FVIII expression was stable for more than half year. The novel msDNA platform with large human FVIII gene expression cassette (2-times larger than AAV capacity) can provide a safe, efficacious, titratable, and redosable genetic medicine for the treatment of HemA patients.

845 Development of Regulatory Cassettes for Gene Therapies Targeting Muscle Stem Cells

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Our group has developed several methods to express therapeutic genes in striated muscle, with a primary focus on establishing effective treatments for Duchenne muscular dystrophy (DMD). These have focused on restoring expression of dystrophin in cardiac and skeletal muscles using Adeno-associated viral (AAV) vector-mediated gene transfer of synthetic dystrophins, or via editing of the endogenous dystrophin gene using CRISPR/Cas9. To ensure efficient and specific expression of AAV delivered genes in postmitotic striated muscles, our approaches utilize in-house developed muscle-specific expression cassettes (MSECs). While resulting dystrophin expression levels and improvements in muscle pathophysiology have been impressive, sustained long-term therapeutic efficacy could benefit greatly from effective co-treatment of muscle stem cells (MuSCs). The therapeutic value of AAV gene transfer to MuSCs has historically been viewed as minimal due to the rapid dilution of therapeutic vector genomes that would occur in dividing stem- and progenitor cells during regeneration of dystrophic skeletal muscle. However, gene editing uniquely enables genomic modifications in MuSCs to be inherited by resulting progeny that either differentiate to repair injured muscle, or return to quiescence as MuSCs for future regenerative needs. Therapeutic MuSC editing is particularly applicable to DMD, due to an abundance of proliferating muscle stem- and progenitor cells responding to continuous bouts of injury and regeneration within dystrophic skeletal muscles. Other groups have reported enhanced therapeutic efficacy and longevity following dystrophin gene editing in MuSCs or progenitors. These studies demonstrate that at least some MuSC populations or progeny thereof can be transduced by AAV vectors, but have relied on ubiquitous and/or non-specific gene regulatory cassettes (RCs) to control editing activity. Non-specific editing approaches are troublesome for future use in patients, not merely based on immunogenicity, but particularly when targeting mitotically competent cells in which unintended editing side-effects could cause detrimental outcomes such as malignancies. MuSC-specific RCs could minimize such outcomes by eliminating potential oncogenic editing of non-target genes or cell types. To address the need for MuSC-active RCs, we have expanded our AAV-size-compatible library of muscle-specific RCs to include RCs with specific activities in muscle stem- and progenitor cells. Here we describe several synthetic RCs derived from highly conserved MyoD1 and Pax7 gene regulatory sequences (two transcription factors involved in the activation or maintenance of quiescent MuSCs). Our studies identify several candidate RCs with preferential activities in myogenic stem- and progenitor cells that are derived solely from MyoD1 or Pax7, as well as from chimeric combinations of regulatory elements from both genes. Safe and effective targeting of MuSCs using promising RC candidates from this study will be instrumental in addressing current limitations of *in vivo* skeletal muscle gene editing, and will help realize the full potential of gene editing for many genetic muscle diseases.

847 Full Characterization of Encapsidated Nucleic Acid Sequence in AAV Manufacturing Using NGS

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Adeno-associated viruses (AAV) are one of the most widely used viral vector-based gene therapy delivery systems in the industry. The manufacturing of such products commonly involves complex steps where a series of 3-4 plasmids are transfected into a producer cell line in order to assemble the viral particles. Each plasmid contains a critical element for viral production, whose product is an AAV particle containing the gene of interest flanked by two inverted terminal repeat (ITR) regions. Due to the nature of AAV particle production and DNA packaging, additional non-targeted sequences may be packaged along with the gene of interest. Even in an optimized system, these non-target nucleic acids can comprise between 1-2% or greater of the entire nucleic acid payload of the particle. These non-target elements generally consist of either host cell DNA or transfected plasmid sequences used for the AAV production. Characterizing the non-targeted sequences that may be packaged in the vector is important for establishing the safety profile of the final product. Although PCR methods directed at detecting common process residual DNA sequences can be adapted for this purpose they may not provide the full information of what is contained in the vector. However, Next Generation Sequencing (NGS) can provide a broader characterization of the non-targeted sequences packaged in the AAV particle. NGS is becoming a widely used tool in biosafety testing strategies for viral and gene therapy products. NGS can be used to create a full genetic profile of all nucleic acids contained within the test sample which can then be assessed using an array of bioinformatic analyses. Vector identity confirmation and variant detection is one of the more common applications, however the data generated by NGS methodology can

provide much more information if mined appropriately. In the case of evaluating encapsidated residual sequences, the AAV sample is first treated with DNase to remove any non-encapsidated residual DNA from the viral preparation. The NGS procedure is then performed on intact viral particles to generate the genetic profile of all encapsidated nucleic acids. After bioinformatic subtraction of the expected AAV sequence, non-target DNA sequences can be assessed and characterized, providing a clearer picture of all encapsidated sequences within the AAV particles. In this presentation the characterization of several representative AAV molecules using this process will be discussed, including the sequencing process, bioinformatic analysis, and representative results. Concepts for the determination of nucleic acid content of full, partial, and empty capsids will also be explored.

848 Safety Profiling of AAV-Based Vectors *In Vitro* - Detection of Differentiated Transcriptomic Signatures in Transduced Primary Human Hepatocytes

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Multifactorial dose-related toxicities following treatments with AAV-based gene therapies have emerged in clinical trials and have raised concerns with regulators and health care providers. The reported adverse events are not limited to specific vectors, although liver toxicities are prominent and have led to discontinuation of clinical trials and serious events caused by already approved products. However, the exact molecular pathologies leading to elevated liver enzymes, serious liver injury, and liver failure remain largely elusive. Clinical safety issues are poorly predicted by testing in preclinical tox species due to differences in biodistribution, transduction efficiencies, payload expression/clearance, and immunogenicity among others. Based on predictive Drug-Induced-Liver-Injury transcriptomics, we set out to build a defined *in-vitro* platform applicable to RNA sequencing of transduced human liver cells, with the aspiration to add molecular insights to vector safety assessments, aided and informed by transcriptomics profiling. Human liver cell lines (HepG2, HepaRG) and primary human hepatocytes (PHH) were seeded in 384-well plate formats and transduced by AAV2, AAV6, AAV8 and AAV9 based vectors expressing a *gfp* or *luc* reporter transgene (N= 8-16/treatment). Control treatments included buffer controls and treatment with empty capsid (N=8-32/treatment). Time-course studies (up to 4 days) and vector titrations (MOI) were used to optimize transduction efficiencies and cell viabilities assessed by quantitative imaging and ATP measurements. All vectors transduced human liver cells, albeit with varying efficiencies (AAV6>AAV2>>AAV8>AAV9) and PHH were selected for transcriptomic profiling. RNA was isolated and subjected to unbiased high throughput RNA-Sequencing (htRNA-Seq). htRNA-Seq was done using ScreenSeq platform, quantifying expression levels for ca. 10,000 genes in a sample. Differential expression and Gene Ontology (GO) enrichment analysis (e.g., assessing stress response and effect on other intrinsic pathways) was done using a ML/AI supported bioinformatics platform (PanHunter). htRNA-Seq enabled reliable phenotype-independent quantification of transgene expression, with sensitivity similar to or higher than sensitivity of e.g., fluorescence-based quantification. More importantly, htRNA-Seq allowed for unbiased analysis of transcriptomic response to transduction with a correlation between MOI and strength of transcriptomic response. A clear separation of response into transgene-independent and transgene-specific components was identifiable in PHH transduced by AAV expressing reporter genes. Enriched GO terms for transgene-independent response components were identified in AAV transduced PHH. In summary, this preliminary study demonstrates that in AAV-transduced PHH quantification of transcriptomic changes provides insight into differentiated responses to AAV vectors and their payloads that can be decomposed in transgene independent and transgene specific components. Further studies will aim to expand this approach into liver micro-tissues and cocultures of mixed cell types with a special view on ML modelling to predict safety liabilities.

849 Single-Base Specific CRISPR Targeting of TERT G228A Mutation with Near-Cognate sgRNA

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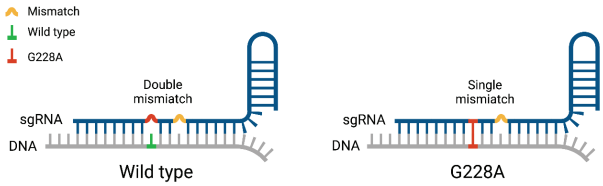
Telomerase regulates the telomeres which are nucleoprotein complexes essential for chromosomal integrity. The telomeres are shortened with each cell division when telomerase is inactivated. In cancer cells, the telomere is maintained by abnormally reactivated telomerase and contributes to cell survival and immortalization. Telomerase Reverse Transcriptase (*TERT*) is activated in cancers through the acquisition of mutation in the *TERT* core promoter region which signifies the most common noncoding mutation. In various cancer types, mostly glioblastomas (80-90%), telomerase is highly expressed dues to overexpression of the *TERT* gene. *TERT* is a druggable and effective target to develop cancer therapeutics.To block telomerase activity, several strategies have been developed including Imetelastat, these have a side effect that can also affect normal cells. To overcome this problem, we address to develop a CRISPR system targeting the *TERT* G228A mutation that promotes cancer development without normal cells. In this study, we deliberately substitute a base of sgRNA to target *TERT* G228A and examined the efficiency of modified sgRNAs through in vitro cleavage screening. Single-molecule analyses showed that the selective binding of the near-cognate sgRNA with a deliberate single-

base mutation to the G228A mutant allele enables the discrimination of the single-base difference. The near-cognate sgRNA also showed highly specific gene editing of the G228A mutant allele in dual fluorescence reporter assessment. Next lentivirus-mediated expression of the near-cognate sgRNA resulted in highly selective targeting of G228A mutant alleles in glioblastoma cell lines, with reduced off-target effects. Together the results suggested the therapeutic potential of CRISPR genome editing with the near-cognate sgRNA.



850 Investigating the Role of Vector DNA Sensing and Transgene Expression on Innate Immune Signaling and Cell Toxicity upon AAV-Mediated Gene Transfer in hiPSC-Derived CNS Models

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Gene therapy with adeno-associated viral (AAV) vectors is being investigated as a promising treatment option for genetic neurodegenerative diseases for which there is currently no cure. Nonetheless, high AAV vector doses are required for clinical efficacy, in particular when targeting the central nervous system (CNS). Recent reports of lethal adverse events and dorsal-root neuron toxicity associated with high AAV doses in clinical trials and large animal studies have raised great concern regarding dose-dependent AAV toxicity and the underlying mechanism. Links between intrinsic innate immunity, cell toxicity and transduction efficacy are emerging, but the potential involvement of innate antiviral sensing of AAV on host-cell toxicity remains largely unknown. Given the species-specificity of vector tropism and innate signaling, and the lack of studies in a human context, we have exploited human iPSC-derived models to assess vector-host interactions and intrinsic innate sensing of AAV in cells of the CNS, in complex environments including mixed neural-glial cultures and 3D cerebral organoids. Functional studies and genome-wide analysis of transcriptional changes triggered by different AAV serotypes revealed early activation of the DNA damage response (DDR), unfolded protein responses and induction of apoptosis, followed by upregulation of inflammatory and antiviral signaling, both in neurons and glial cell subsets. Innate responses and cellular toxicity were further enhanced in mixed cultures and organoids, analyzed by single-cell RNAseq, as compared to the transduction of the various cell types individually, suggesting an active interplay between the different CNS cell subsets in the establishment of a detrimental inflammatory environment. Finally, we used vectors carrying different promoters and transgenes in order to dissect the contribution of the DDR, transgene expression and transgene sequence respectively on the signaling observed in organoids analyzed by single-cell RNASeq. Together, our studies shed light on the mechanisms leading to AAV sensing and neurotoxicity, informing the development of more effective gene therapy and gene correction strategies.

851 Immunogenicity of SARS-CoV-2 DNA Vaccine Candidates Formulated with the Fusion-Associated Small Transmembrane Protein Proteolipid Vehicle Delivery System

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DNA vaccines have had limited success in the clinic despite decades of research and development, owing primarily to challenges in delivery. Here we report the generation of DNA vaccine candidates against SARS-CoV-2 utilizing an intracellular nucleic acid delivery platform, FAST-PLV, where plasmid DNA is encapsulated in proteolipid vehicles formulated with a fusion-associated small transmembrane protein and well-tolerated lipids utilizing a scalable microfluidic approach. Selected SARS-CoV2 vaccine candidates comprising full-length SARS-CoV-2 Spike protein combined with two genetic adjuvants (CpG motifs, RIG-I agonist, termed NP-S) elicited potent neutralizing antibody responses in mice and non-human primates at low (sub-milligram) doses indistinguishable from an approved mRNA vaccine. A plasmid DNA vaccine candidate encoding full length Spike protein, NP-S, stimulated significant spike-specific T cell responses including functional cytotoxic T lymphocyte responses. A single dose of NP-S protected hamsters from morbidity following SARS-CoV-2 challenge. The FAST-PLV vaccine platform enables efficient intracellular delivery of DNA payloads and is ideally suited for the development of fridge-stable genetic vaccines against emerging infections.

852 Gene Therapy Approach for Hereditary Spastic Paraplegia Type 52 (SPG52)

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Hereditary spastic paraplegia type 52 (SPG52) is an ultra-rare inherited neurological disorder characterized by lower limb spasticity, weakness, global developmental delay, intellectual disability, and seizures. SPG52 is caused by a biallelic mutation in AP4s1 gene, which encodes a subunit of the adaptor protein complex 4 (AP-4). Mutations in any of its 4 subunits result in complex destabilization and degradation of all subunits, leading to a shared pathophysiology and symptomatology. The function of AP-4 within the cell, although unclear, it is believed to play a key role in autophagosome generation. Currently, this disease does not have a cure nor treatment. We believe that gene therapy targeted to restore AP4s1 expression is a rational therapeutic approach to ameliorate the disease phenotype. Thus, we have generated, characterized, and successfully treated patient-derived fibroblasts with viral vectors carrying a correct copy of the AP4s1 gene. This treatment has not only restored the expression of this specific subunit but has enabled complex expression restoration. To further study the effects of our gene therapy approach in a disease relevant cell type, we have developed and characterized iPSC-derived cortical neurons from patient's samples, which display autophagy alterations. In parallel to our *in vitro* studies, we have developed the first rodent animal model of SPG52. This animal model is a knock-out (KO) mouse generated using the CRISPR-Cas technology targeted to abolish AP4s1 expression in a C57Bl6 strain. To characterize the animal model, cognitive and physical skills batteries have been performed. KO mice show decreased motor coordination as well as muscle strength by grip strength, inverted grid, RotaRod and clasping tests. These KO animals, based on their performance doing the Open Field and Morris Water Maze tests, also display impaired memory while having a regular exploratory behavior with no signs of anxiety. Neuroanatomical changes described in patients, such as the thinning of the Corpus Callosum, have been observed by magnetic resonance imaging and confirmed by histology. In the present, we are treating AP4s1 KO animals systemically with *in vitro* validated neurotropic AAV vector carrying a correct copy of the AP4s1 gene at different ages and assessing phenotype rescue with the battery of cognitive and physical skills tests. The generation of the hiPSC model together with the establishment of this newly generated mouse model, with a phenotype that resembles the human pathology, will be crucial to elucidate SPG52 pathogenesis and to validate our gene therapy approach, key milestones on the road to a successful treatment for SPG52.

853 Engineering Inducible Signaling Receptors to Increase Erythropoietic Output from Genome-Edited Hematopoietic Stem Cells

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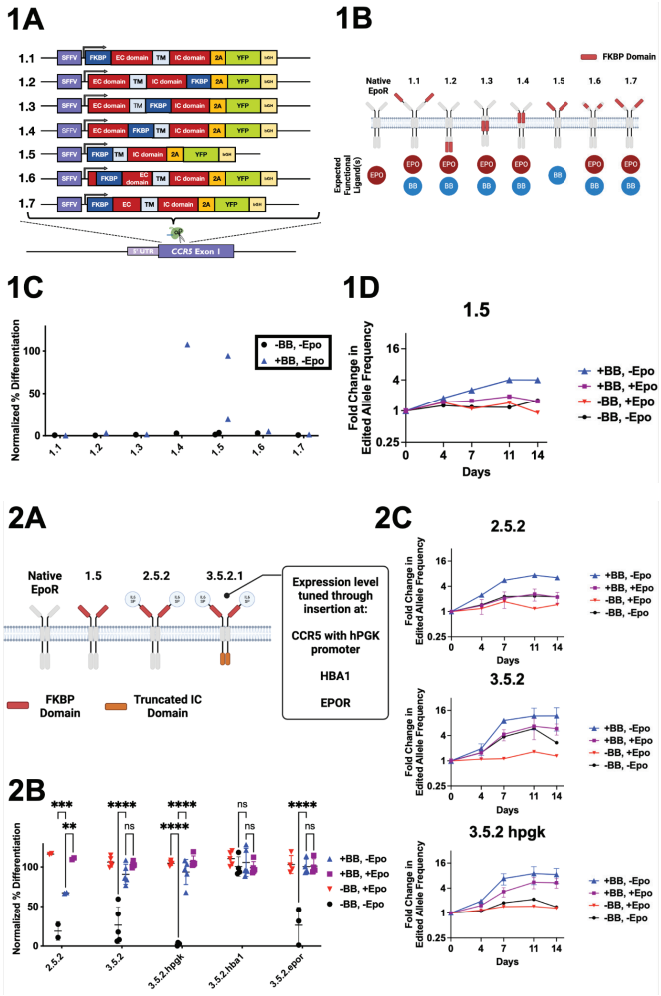
Introduction: Cell and gene therapies (CGTs) are classically limited by editing and delivery efficiencies. However, for hematopoietic stem cell (HSC) therapy, differentiation of the edited stem cell population toward the disease-relevant lineage also has a great bearing on the degree of phenotypic effect. While some diseases exert a selective advantage for genetically modified cells based on the underlying disease pathophysiology, many do not. Although we understand the pathways that define such lineage choices, directly dosing with signaling receptor ligands would not confer selective enrichment of edited cells. Moreover, supra-physiologic dosing with such factors may have deleterious effects on the homeostatic balance of stem cell differentiation. Thus, we designed a system to selectively influence edited cell fate without acting on the broader HSC compartment. Here, we present a set of chimeric, synthetic receptors that enrich edited cell populations towards the erythroid lineage via a non-immunogenic, non-toxic small molecule (SM). **Methods:** In vitro editing was performed using Cas9 protein complexed with the desired gRNA introduced via electroporation and an AAV6 DNA repair template that could be integrated via HDR. Editing was conducted in primary human CD34+ HSCs. Differentiation was determined by flow cytometry assessment of red blood cell (RBC) surface markers. Allele frequency of the edited population during differentiation was determined via droplet digital PCR. **Results:** We hypothesized that inducible dimerization domains currently in the clinic might be paired with EPOR in order to place downstream signaling under the control of a bioavailable SM we refer to as "BB". To test this, we designed a series of FKBP-EPOR chimeras (Fig 1a,b) that are able to yield BB-induced erythroid differentiation (Fig 1c,d). We refer to these constructs as RBCellDrives. We then optimized the design of the RBCellDrive via a) addition of a highly efficient secretory signal peptide; b) inclusion of a naturally occurring intracellular domain truncation with enhanced erythropoietic capacity; and c) insertion with different promoters and at different loci to observe the possible range of expression levels and kinetics (Fig 2a). Through construct optimization, the potency and fidelity of RBC differentiation under BB stimulus ultimately matched or exceeded the ability of EPO (Fig 2b). This was further evidenced by the frequency of edited cells increasing dramatically over the course of RBC differentiation in a BB-dependent manner (Fig 2c). **Conclusions:** We demonstrate the development of synthetic receptors—RBCellDrives—that inducibly mimic the EPOR signaling cascade with high fidelity while obviating dependence on EPO for erythropoiesis. In sum, this could allow clinicians to non-invasively boost the production of clinically meaningful RBCs post-

transplantation. Our work, therefore, has the potential to dramatically improve the efficacy of CGTs and has created novel tools that can be used to better understand fundamental stem cell biology.



854 Use of Precision-Cut Lung Slices to Evaluate Lung Gene Therapy Vectors

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Cystic fibrosis (CF) is the most common monogenic fatal genetic disorder in North America, with a prevalence of around 1 in 2500 people. CF is caused by a diverse range of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that lead to systemic disease, with the most fatal pathology occurring in the lung. Gene therapy offers a promising alternative to current treatments as it would work for all CF patients, regardless of mutations. The primary target for gene therapy would be the lung. To ensure the most relevant gene therapy vectors are selected, precision-cut lung slices (PCLS) can be used as an early model to screen candidates. PCLS retain the cellular architecture, microenvironment, and variety of cells of the lungs. **Objective**: The objective of this work was to determine if PCLS transduction can accurately predict *in vivo* transduction results. **Methods**: In this work, the gene therapy vectors Adeno-associated virus (AAV) (serotype AAV6.2FF and AAV9) and lentivirus carrying either green fluorescent protein (GFP) or alkaline phosphatase (AP) as the reporter gene were used to transduce PCLS generated from mice, ferret, sheep, and pigs. Using microscopy and flow cytometry, GFP expression was measured. Using a visual stain and activity assay, AP expression was measured. Animals were also administered the same vectors intranasally, and GFP or AP expression was measured. **Results**: Mouse PCLS showed much higher transduction with AAV6.2FF-GFP than AAV9-GFP via microscopy. This was consistent with *in vivo* transduction results, where flow cytometry demonstrated AAV9-GFP only transduced 25% of the target lung cell population, whereas AAV6.2FF-GFP transduced 65%. In sheep PCLS, AAV6.2FF also outperformed AAV9 in GFP expression. *In vivo*, AAV6.2FF trended towards higher AP expression in mouse lung than AAV9. This higher AP expression was also demonstrated on pig PCLS using the AP stain. Ferret PCLS and lentiviral results are pending. **Conclusion**: PCLS results closely predicted *in vivo* results, and will provide a valuable model on which to screen lung gene therapy vector candidates.

855 Developing an AAV Affinity Chromatography Step

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Cost reduction remains a challenge for downstream processing of recombinant adeno-associated viruses (rAAV). The current work focuses on improvement in recovery through optimization of the elution conditions for the affinity capture step using the commercially available AVIPure® AAV9 resin and an early prototype AVIPure® AAV5 resin. The effect of elution pH, conductivity, type of salt, and additives are demonstrated using a design of experiments (DoE) approach. Such experiments can greatly improve the yield of the chromatography step, while also enabling elution at a higher pH to alleviate concerns regarding viral stability.

856 Silicon Nanoneedle-Mediated Cargo Delivery for Cell Engineering

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Mekonos, San Francisco, CA

Techniques to introduce foreign molecules or materials into living cells are crucial and potentially rate-limiting processes for cell engineering. Innate cellular functions can be artificially manipulated and reprogrammed by introducing biomolecules such as DNA, RNA, and proteins into the cytosol or nucleus. This process of introducing exogenous cargo into living cells is called intracellular delivery. One popular type of cargo for intracellular delivery is CRISPR gene editing technology, which typically requires delivery of two main components: a guide RNA targeting a specific genomic locus and an RNA-guided endonuclease, such as Cas9, that creates site-specific double-stranded DNA breaks (DSBs) and enables genomic modifications. For certain genome editing applications (i.e. knock-ins), a template plasmid, to facilitate homology-directed repair (HDR), is also required to be delivered into target cells. A big barrier to intracellular delivery

is crossing the cell membrane. Traditionally, viral vectors and electroporation tools have been used to deliver foreign biomolecules into cells. However, both viral vectors and electroporation have limitations and are not optimal for achieving high levels of intracellular delivery while simultaneously maintaining cell viability and functions. As new solutions, microfluidic and nanoneedle approaches show exceptional potential for addressing these unsolved cell engineering obstacles. In this study, we show that Mekonos' silicon nanoneedle-based delivery platform demonstrates unique capabilities for high-efficiency cargo delivery, including CRISPR/Cas9, while maintaining cell viability for cell engineering.

857 Plasmid Impurity Sizing of the *nptII* Kanamycin Resistance Gene in Recombinant Adeno-Associated Virus by Droplet Digital Polymerase Chain Reaction

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Introduction: Recombinant adeno-associated virus (rAAV)-encapsidated plasmid impurities are an undesirable byproduct of vector manufacturing consisting of heterogeneous (non-vector genome) fragments of DNA arising from the production plasmids. Regulatory authorities require thorough quantification of DNA impurities, but current qPCR methods used to detect impurities are based on quantification of small fragments of the impurity species being quantified. Therefore, these methods provide very limited information on the size distribution of mispackaged DNA species. Horizontal gene transfer of manufacturing plasmid-derived resistance genes is a potential risk when they are packaged as full-length genes. However, this risk may not be identified by currently available analytical methods. Here, we describe a novel method for assessing the size profile of plasmid-derived antibiotic resistance gene impurities in rAAV products. **Method:** A droplet digital PCR (ddPCR) assay was developed to assess kanamycin resistance gene (*nptII*) fragment sizes using four sizes ranging from 99 bp to 795 bp. To measure smaller fragments up to 495 bp in length, a singleplex ddPCR method was used with two primers and one probe. Two duplex ddPCR approaches were used to analyze the full-length gene. The first approach used two primer-probe sets with different fluorophores positioned 795 bp apart so that droplets positive for both fluorophores indicate full-length *nptII* gene sequences. However, this approach carries the risk of overestimating full-length DNA species if two smaller *nptII* gene fragments are co-packaged in the same rAAV capsid, creating double-positive droplets. To reduce false positives, a second approach was developed using two primer-probe sets with two different fluorophores spanning approximately 400 bp fragments at each side of the gene, minimizing the risk of false-positive full-length results (Figure 1). Acceptance criteria for the assay included recovery of >90% of a linearized plasmid control for all fragments analyzed, confirmation of dilutional linearity for plasmid controls and samples, and coefficients of variation <15% for replicate measurements. **Results:** Four large-scale rAAV batches using three different expression cassettes were analyzed for *nptII* impurities using this method. Across batches, the packaging frequency of impurities correlated inversely with fragment length, consistent with long-read next-generation sequencing data. Additionally, batches produced with the same manufacturing platform had consistent size profiles, regardless of the expression cassette. **Conclusions:** The method described here meets requirements for analysis of the size profile of packaged kanamycin resistance gene impurities. Furthermore, the assay enables the assessment of the effects of different biological starting materials and process parameters on the *nptII* impurity size profile. The data generated from this method can form part of a comprehensive assessment of the potential risk from packaged plasmid impurities.

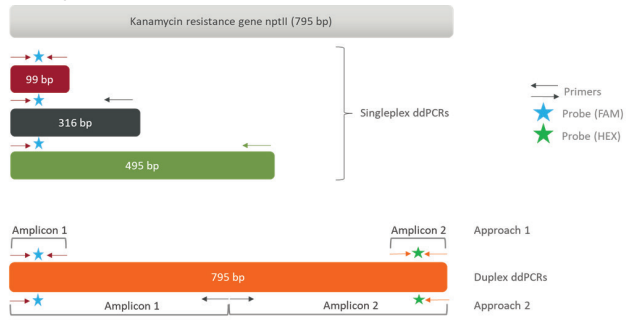

Figure 1. Schematic representation of the assay principle of singleplex and duplex ddPCRs for *nptII*.

858 High-Throughput Pooled Screen of CAR Library Identifies Essential Signaling Features of CAR-T Cells That Resist Immunosuppression

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While T cells engineered with canonical chimeric antigen receptor (CAR) designs achieved clinical success in hematological cancer indications, they have shown limited efficacy against solid tumors due to lack of persistence and susceptibility to various immunosuppressive factors in the tumor microenvironment (TME). Systematic discovery of CAR architectures that elicit enhanced T cell functional phenotypes under immunosuppressive environments has been challenging due to technical constraints in high-throughput screening and scaling. Here, we generated a lentiviral CAR intracellular domain (ICD) library composed of 10,000 different 2nd generation CAR designs with individual DNA barcodes in both low and high affinity ROR1 scFv binders and screened for T cell persistence in a chronic stimulation model in a pooled manner. We found unique ICD hits across different binders that outperformed canonical CAR ICDs, which were subsequently validated via an arrayed screen. In order to identify CAR ICDs that potentially resist exhaustion and TME suppression, we screened individual ICD hits in the presence of TGFβ and adenosine against 3-D spheroids in a hypoxic environment. Functional characterization data sets including cytotoxicity, cytokine profiles, and transcriptomics allowed us to comprehend essential signaling components of CAR that could resist a variety of immunosuppressive factors and maintain memory phenotype of T cells for a prolonged period. We believe our approach is readily applicable to identifying signaling

modules driving favorable phenotypes of diverse immune cell types against solid tumors. With the growing interest of utilizing non-conventional T cell subsets, such as γδ T or NKT cells, NK cells, and macrophages as platforms for cell therapy, our CAR library and high-throughput screening platform will facilitate discovering optimized architectures of CAR molecules that could benefit patients with diverse indications in the clinic. Additionally, our platform is compatible with engineering other aspects of CARs including extracellular domains and armoring features of immune cells, such as decoy or switch receptors, that could further assist bypassing TME suppression.

859 Multiplexed Optimization of Serotype, Regulatory Elements and AAV Genome in a Large Animal Model of Heart Failure

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Gene therapy has had remarkable success in treating rare, monogenic diseases. Realizing this potential for diseases without a clear genetic cause will rely on new methods for identifying optimal molecular targets with demonstrated efficacy. Heart failure is a complex genetic disease that involves the interplay of different cell types and contributions from the systemic environment, meaning that therapeutic efficacy is ultimately only testable in vivo. To increase the scale at which targets can be validated *in vivo*, we previously developed and validated a screening platform where many AAV gene therapies are delivered simultaneously, in a single animal, using single-cell genomics as a phenotypic readout for the therapeutic efficacy of each candidate. In order to apply our platform to heart failure, we required the development of a delivery modality, such as AAVs, capable of cell-type specificity and high transgene expression in the most relevant model, the porcine aortic banding model. Seventeen AAV serotypes were pooled and injected into two mice at doses of 3E10 vg/serotype and 1E11 vg/serotype. After procuring the heart tissue, isolating cardiomyocytes, and running through 10x genomic single-cell workflow, we were able to sequence and process data with readouts of serotype internalized into single-cells. This allowed us to identify several top AAV serotypes: AAVMYO, AAV9, and AAV1 that were validated in individual mice with histological and flow cytometry readouts. Additionally, we tested the top promoters that were identified using a similar single-cell RNA barcode screen of 47 unique regulatory element combinations. We used this data to inform generation of a library for the porcine aortic banding model of heart failure. Seven unique constructs comprising multiple serotypes, regulatory elements, and both single- and double-stranded AAV genomes, were delivered two weeks following aortic banding at a total dose of 2E14 vg. Four weeks later, histology and single cell sequencing allowed us to identify the optimal serotype, regulatory elements and AAV genome configuration in this large animal model. Having optimized gene delivery in the most relevant model of heart failure, our next steps are to use our platform for multiplexed screening of hundreds of therapeutic candidates for this non-monogenetic, complex disease.

860 Local Delivery of Vectorized Therapeutics Targeting a Pro-Inflammatory Cytokine Effectively Reduces Disease Severity in a Mouse Model of Non-Infectious Uveitis

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Uveitis comprises a heterogenous group of inflammatory intraocular diseases that can result in vision loss. Non-infectious uveitis (NIU) of the posterior segment of the eye can be difficult to treat, requiring long-term systemic immunosuppression with significant side effects. Here, we test the hypothesis that local expression of cytokine inhibitors in the eye, mediated by AAV delivery, can suppress inflammation as a potential treatment for uveitis. Inhibitors of the pro-inflammatory cytokine TNF-α, a validated target for treating uveitis, were evaluated in multiple formats including modified soluble receptors and antibodies. Vector expression was initially screened *in vitro* via transfection in 293T and ARPE-19 cells. Two additional cell-based assays were used to test inhibitory activity across multiple species. Inhibitors demonstrating high expression and bioactivity were then produced as AAV8 vectors for further testing *in vivo*. The classical B10.RIII Experimental Autoimmune Uveitis (EAU) mouse model was used to test whether local expression of anti-TNF-α factors was sufficient to prevent intraocular inflammation. Three weeks after subretinal injection of AAV, EAU was induced. Ocular function and disease progression were assessed at multiple timepoints using optical coherence tomography, fundus imaging, and optokinetic tracking. Following final *in vivo* assessments, eyes were collected and examined further for EAU histopathology or transgene expression. Clinical scores were assigned using established criteria for evaluation of fundus and histopathology images. Control EAU animals receiving either vehicle or a non-specific transgene demonstrated a robust increase in ocular inflammation as indicated by high clinical scores and near complete absence of visual acuity. In animals expressing potent TNF-α inhibitors, we observed a dose-dependent reduction in the severity of inflammation and improvement in visual acuity. These data suggest that local inhibition of TNF-α via AAV-mediated transfer of an inhibitor in the eye can successfully limit inflammation associated with uveitis and support further pre-clinical development of a vectorized therapeutic approach to the treatment of non-infectious uveitis.

861 Trivalent miRNA Constructs Targeting C9ORF72 for the Treatment of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are severe neurodegenerative diseases with no effective treatment. ALS is a fatal neurodegenerative disease characterized clinically by progressive paralysis leading to death from respiratory failure, typically within three to five years of symptom onset. A hexanucleotide GGGGCC repeat expansion in the C9ORF72 gene is the most frequent genetic cause of both ALS and FTD in Europe and North

America. The mechanisms of C9ORF72 toxicity in neurodegeneration is suggested by loss of function of C9ORF72 protein, and toxic gain of function from sense and antisense C9ORF72 repeat RNA or from dipeptide repeat proteins (DPRs). In this study, we designed trivalent constructs comprised of C9ORF72 gene supplementation and miRNA cassettes to knockdown C9ORF72 sense and antisense transcripts. We designed 86 miRNAs that target both sense and antisense C9ORF72 transcripts. To screen and select the top sense and antisense microRNA candidates that cause the C9ORF72 transcript knockdown, we utilized the dual luciferase reporter assay after co-transfecting miRNA plasmids and reporter plasmid into HEK293T cells. Based on reporter screening results, we performed bioinformatics analyses to predict top 30 miRNA target sites, binding potential among species, and identify off-targets. Next, we confirmed the top 25 miRNA candidates' performance in vitro using RT-qPCR analysis on the endogenous C9ORF72 by transfecting the miRNA plasmids into HEK293T cells. The top 10 best performing miRNA were chosen to build the trivalent constructs. Each construct contains sense and antisense miRNA under pol III promoter and a C9ORF72 gene that is resistant to the miRNA (codon-modified non-self-silencing C9ORF72). We utilized the luciferase assay to confirm the miRNA knockdown effects and western blotting to verify the expression of C9ORF72 protein from the miRNA resistant constructs. We identified 19 miRNAs targeting a variety of loci along C9ORF72 with at least 70% knockdown using luciferase reporter assay. Bioinformatics revealed the top 5 sense miRNAs and 1 antisense miRNA that have preferred target sites on canonical C9ORF72 gene with least off-target predictions. RT-qPCR showed the top miRNAs reduced endogenous C9ORF72 mRNA levels in HEK293T cells by at least 50%. Trivalent construct performance in the reporter assay showed consistent knockdown effects of the miRNA. Western blot analysis verified the expression of C9ORF72 from the trivalent miRNA resistant constructs. In this study, we report for the first time the generation of trivalent constructs containing miRNA to silence endogenous C9ORF72 and augmenting C9ORF72 levels using miRNA resistant constructs, making them a potential therapeutic approach to target ALS. The next steps will be investigating the effect of AAV containing the trivalent constructs in human-derived iPSC neurons, transgenic C9ORF72 mouse model and non-human primate to facilitate the effective treatment for ALS and FTD patients.

862 A Novel, Highly Efficient Epigenome-Editing System Based on an All-in-One Adeno-Associated Vector Delivery Platform: Preclinical Proof-of-Concept and *In Vivo* Validation

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Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/dCas9-based epigenetic editing is an emerging field in developing safe and effective gene engineering tools. The system consists mainly of a single guide RNA (sgRNA) and a deactivated-Cas9 nuclease (dCas9). By fusing a transcriptional repressor domain to the CRISPR/dCas9 nuclease, transcriptional repression complexes are recruited to silence the specific gene target sites safely and effectively. Adeno-associated vector (AAV) is a platform-of-choice for the delivery of therapeutic cargoes; nevertheless, its small packaging capacity is mainly not suitable to be utilized for the efficient delivery of large cargoes, such as CRISPR/dCas9-effector systems. To circumvent this limitation, most AAV -based CRISPR/Cas tools delivered separately from two viral cassettes. However, this approach requires higher viral payloads and usually is less efficient. Here, we describe a compact CRISPR/dCas9 system packaged within a single AAV vector. The system based on small transcription repression domain (TRD) derived from MeCP2 repressor, fused with KRAB repressor. We demonstrated that the developed system can be efficiently packaged into AAV particles. Using this system, we were able to induce a robust and sustained editing in vitro and in animal models of neurological diseases in vivo. We believe that the novel AAV-based platform could broaden the CRISPR/Cas9 toolsets for efficient epigenome-based perturbations in the therapeutic settings.

863 Low-Dose Binary Oncolytic/Helper-Dependent Adenovirus (CAdVEC) Promotes Anti-Tumor Activity in Preclinical and Clinical Studies

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Administration of oncolytic adenoviruses may help control some human tumors, but their limited potency means toxic or technically unfeasible doses may be required for maximal efficacy. Here we show that a binary oncolytic/helper-dependent adenovirus (CAdVEC) that both lyses tumor cells and locally expresses the pro-inflammatory cytokine IL-12 and PD-L1 checkpoint blocking antibody has high potency and efficacy in a range of humanized mouse models, including breast and pancreatic cancers. Based on these preclinical studies, we treated 4 patients with relapsed, resistant head and neck squamous cell cancer or breast adenocarcinoma (NCT03740256) with a single intratumoral injection of a low-dose of CAdVEC, representing a quantity of oncolytic adenovirus that is more than 100-fold lower than the starting dose of previous oncolytic adenovirus trials. While low-dose CAdVEC caused no significant hepatic transaminitis or systemic cytokine elevation, it nonetheless induced proinflammatory repolarization of the tumor microenvironment with increased infiltration of natural killer cells and CD8 T cells, replicating the effects observed in our humanized mouse models. A single intratumoral administration of low-dose CAdVEC was associated with both loco-regional and abscopal effects on distant metastases, and with subsequent administration of systemic immune checkpoint inhibitor, induced long-term anti-tumor effects, including one complete and two partial responses in patients. In both pre-clinical and clinical studies, a binary combination of oncolytic and immunostimulatory adenoviral vectors (CAdVEC) is safe and sufficiently potent to induce significant tumor control through oncolysis and immune repolarization, even at

extremely low doses. We are now investigating the mechanism(s) by which such low-dose CAdVEC led to durable anti-tumor responses in patients.

864 A Dual-Fluorescence Assay for Gene Delivery Vehicle Screening in Macrophages with an Inflammation-Inducible Reporter Construct

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Macrophages are necessary for the immune system to respond appropriately to outside invaders. Macrophages are complex and can change phenotype according to specific activation pathways. This includes an array of activation states from homeostasis to defense. When cellular pathways that affect macrophage plasticity are dysregulated in disease, macrophages can remain in their inflammatory state, and drive chronic inflammation. The role of macrophage plasticity in chronic inflammation is an interesting target for therapeutic development in chronic inflammation. One issue with targeting macrophages for drug development is their innate response to outside invaders, which makes delivery using viral or non-viral vehicles difficult and can cause activation of macrophages. Macrophages have been targeted in the past for cancer cell therapy where the macrophages needed to be activated to attack the cancerous cells. In an environment where the goal is to diminish inflammation, a delivery vehicle that both ensures efficient delivery and mitigates inflammatory response. We propose the development of an inflammatory reporter plasmid that expresses mCherry in an inflammatory environment. When activated, macrophages produce the inflammatory cytokine TNF-α which in turns activates NF-kB, a potent transcription factor. The inflammatory reporter plasmid utilizes mCherry under a previously defined inflammatory promoter that recruits NF-kB regulatory elements to a minimal CMV promoter that acts as the transcription start site. The reporter was then stably integrated into RAW264.7 mouse macrophages to test different viral and non-viral delivery vehicles. Various serotypes of the viral vectors Adenovirus (AdV), Adeno-associated virus (AAV), and lentivirus were used to deliver green fluorescent protein (GFP) to the RAW264.7 inflammatory reporter cell line. Using this dual-reporter system, we can determine which delivery vehicles provide both efficient delivery and minimize the inflammatory response by macrophages. Number of cells expressing mCherry and GFP will be quantified using flow cytometry and the output will be a matrix where GFP+/mCherry- equates to efficient delivery and minimal/no inflammation. Development of this dual-reporter assay will establish an effective delivery method that can be utilized as a starting point for a macrophage therapeutic platform, the basis for high-throughput screening, and manipulation of macrophages *in vivo*.

866 Anti-PD-1 Chimeric Antigen Receptor T Cells Efficiently Target SIV-Infected CD4+ T Cells in Germinal Centers of Rhesus Macaques

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Background: Programmed cell death protein 1 (PD-1) is an immune checkpoint marker commonly expressed on memory T cells and enriched in latently-infected CD4+ T cells containing replication-competent human immune deficiency virus 1 (HIV) provirus in people with HIV on antiretroviral therapy (ART). **Methods:** We engineered novel chimeric antigen receptor (CAR) T cells that can efficiently kill PD-1 expressing cells *in vitro* and *in vivo* to assess the impact of PD-1 depletion on viral reservoirs and rebound dynamics in simian immunodeficiency virus (SIV) mac239-infected rhesus macaques (RMs). Adoptive transfer experiments of anti-PD-1 CAR T cells were done in 2 SIV naïve and 4 SIV-infected RMs on ART. **Results:** In 3 of 6 RMs, one SIV naïve and 2 SIV+ RMs, anti-PD-1 CAR T cells expanded efficiently and persisted for up to 100 days concomitant with the depletion of PD-1+ memory T cells in blood and tissues, including CD4+ follicular helper T cells (T_{FH}). This depletion of T_{FH} in lymph nodes was also associated with depletion of detectable SIV RNA from the germinal center (GC). However, following ART interruption, there was a marked increase in SIV replication in extrafollicular portions of lymph nodes, a 2-log higher plasma viremia relative to controls and accelerated disease progression, associated with the acute depletion of CD8+ memory T cells after CAR T infusion in SIV+ RMs on ART. **Conclusions:** These data indicate anti-PD-1 CAR T cells can target and deplete PD-1+ T cells *in vivo* including GC T_{FH} cells and eradicate SIV from this immunological sanctuary. Approaches to limit CAR T cell-mediated depletion to PD-1+ CD4+ T cell reservoirs and reduced off target CD8+ memory T cell depletion should be pursued.

867 Potent and Uniform Fetal Hemoglobin Induction via Base Editing

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Elevated fetal hemoglobin (HbF, α2γ2) in red blood cells (RBCs) can ameliorate sickle cell disease and β-thalassemia. Gene editing strategies are a promising approach to manipulate the regulatory genetic elements and induce therapeutic levels of HbF. Base editors have been used to generate naturally occurring hereditary persistence of fetal hemoglobin (HPFH). Moreover, HPFH variants that create de novo binding motifs for erythroid transcriptional activators at the γ-globin promoter can be introduced with base editors but not with Cas9 nuclease mediated non homologous end joining. However, the best gene editing strategies for therapeutic HbF induction are not known.We compared Cas9 nuclease and base editing strategies for HbF induction by modifying $CD34^+$ hematopoietic stem and progenitor cells (HSPCs). Specifically, we used Cas9 nuclease to disrupt an erythroid enhancer in the *BCL11A* gene or a BCL11A binding motif in the γ-globin promoter. Alternatively, we used adenine base editors to create base pair substitutions at γ-globin promoter positions -198, -175, and -113, thereby generating de-novo binding motifs for KLF1, TAL1, and GATA1, respectively. The -175 A>G base edit proved to be the most potent edit.Clonal analysis by analyzing burst forming unit-erythroid (BFU-E) colonies derived from base edited CD34+ cells revealed that -175 A>G base edit in all four γ-globin genes expressed 81±7% HbF compared to 17±11% in unedited controls. HbF induction in BFU-E colonies was lower and more variable with two Cas9 nuclease strategies: 32±19% and 52±13% HbF expression after targeting the γ-globin promoter and *BCL11A* enhancer, respectively. Surprisingly, analysis of specific indels in colonies revealed significant HbF induction with a 13 bp and 4 bp deletion but not with -1, -2, -3 or +1 indels, even though they all disrupted the BCL11A binding motif. In contrast, all high frequency indels targeting the *BCL11A* enhancer disrupted a functionally important GATA1 binding motif and induced HbF similarly, albeit with greater variability than colonies with the -175 A>G base edit.Installing the -175 A>G base edit with the more active ABE8e editor yielded a 1.6-fold improvement in editing efficiency relative to ABE7.10, accompanied by 1.4-fold increase in %HbF induction. In xenotransplant studies with ABE8e using HSPCs isolated from an SCD patient, we achieved on-target input editing of 62% and retained 42±3% after engraftment, resulting in 62±8% HbF expression. After transplantation of $CD34^+$ cells from the same donor that were treated with Cas9 nuclease to disrupt the BCL11A binding motif, 27±5% HbF was achieved with 75±5% editing in engrafted cells. Overall, our data identifies a base editing strategy for potent induction of HbF and demonstrates that diverse indels generated by Cas9 nuclease cause unexpected variation in biological outcomes that can be circumvented by base editing, with important implications for therapeutic gene editing efforts.

868 Multilineage Engraftment of HSCs Derived Anti HIV CAR Cells *In Vivo*

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Achieving HIV cure or remission without anti-retroviral therapy (ART) treatment requires the enhancement and persistence of effective antiviral immune responses. Chimeric Antigen Receptor (CAR) T-cells have emerged as a powerful immunotherapy for various forms of cancer and show promise in treating HIV-1 infection. However, persistence, trafficking, and maintenance of function remain major challenges for peripheral CAR-T cell therapy, which results in limited antiviral efficacy in vivo. To overcome those limitations, we created long-lasting anti-HIV responses by modifying autologous Hematopoietic Stem Cells (HSCs) with anti-HIV CAR molecule. In addition, the gene therapy vector expressing this CAR molecule also contains anti-HIV genes, which protects the CAR-expressing immune cells from infection. Our studies in humanized mice and non-human primates (NHPs) have demonstrated that HSCs based CAR therapy could provide life-long engraftment and production of functional CAR-T cells, resulting in significantly reduced viral rebound after ART withdrawal. Interestingly, we observed successful differentiation of multiple hematopoietic lineages expressing CAR molecule, including CAR macrophages and CAR-NK cells that could contain anti-HIV activity and boost functions of CAR-T cells. Importantly, we demonstrated that HSC-derived, multilineage CD4CAR+ cells were able to traffic to and persist in HIV tissue associated reservoir sites, including lymphoid germinal centers, central nervous system tissues, and gastrointestinal tract tissues. To further investigate the antiviral functions of multilineage CAR immune cells, we transduced primary NK cells and Macrophages with improved second generation anti-HIV D1D2CAR-41BB lentivirus used in our previous studies. We found that both CAR-NK and CAR-MQs secrete proinflammatory cytokines and kill HIV+ target cells. To further investigate CAR T and CAR NK cells in vivo, we generated humanized mice using human CD34+ HSCs that were gene modified with D1D2CAR-41BB in NSG-Tg(Hu-IL-15) mice. We observed successful CAR T cells and CAR NK cells differentiation from modified HSCs in NSG-hu-IL-15 mice. After HIV infection, we detected significant increase of activation receptors NK2GD and NKp46 on CAR-NK cells. Importantly, a significant (>6 fold) reduction of viral load in D1D2CAR-HSCs mice were observed as early as two weeks post infection. The early viral suppression may

be contributable to CAR-NK cell activity as most naïve CAR-T cells have not fully differentiated into effector T cells yet. Collectively, our data support that development of multi-armed immunity from CAR engineered HSCs may provide broader and long-term immune surveillance for HIV infection.

869 Persistent Genetic Modification of K562 and Human Hematopoietic Stem and Progenitor Cells Using S/MAR DNA Nanovectors

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Effective strategies for the persistent genetic modification of hematopoietic stem and progenitor cells (HSPCs) have the potential to revolutionize the treatment of various genetic hematological disorders like severe combined immunodeficiency and Fanconi anemia. For gene delivery into HSPCs recombinant integrating viruses are a potent and widely used tool. However, their random integration into the genome introduces a risk of insertional mutagenesis. Their innate immunogenicity also presents another impediment to clinical application. To achieve the goal of an efficient and safe generation of stably transfected cells of a variety of cell types, we have developed a non-viral DNA vector platform based on Scaffold/Matrix-Associated Regions (S/MAR). The episomal maintenance of S/MAR DNA nanovectors (nS/MAR) eradicates the risk of insertional mutagenesis, providing a major advantage compared to already clinically applied approaches using CRISPR/Cas9 systems or viral vectors. Additionally, with its unlimited capacity, even large therapeutic transgenes or other sophisticated genetic elements can be delivered with nS/MAR. Furthermore, genetic engineering based on n/SMAR with its inherent mitotic stability should minimize costs for the manufacturing of genetically modified HSPCs. In this study, we have evaluated the efficiency and persistence of reporter transgene expression in the widely used hematopoietic K562 cell line and HSPCs using nS/MAR. K562 cells and primary human HSPCs were electroporated with nS/MAR containing the human elongation factor-1 alpha promoter and a fluorescent reporter transgene (dTomato) using the MaxCyte ExPERT® platform. HSPCs were isolated by performing MACS® Cell Separation with CD34 microbeads on 10-year cryopreserved leukapheresis material. 2.5 x 10^6 K562 cells and 1.25 x 10^6 primary human HSPCs were electroporated using the MaxCyte ExPERT GTx®. Transfection efficiency and cell viability were analyzed using flow cytometry. Our study demonstrates the feasibility of using nS/MAR for persistent genetic modification of K562 and HSPCs. K562 cells, which serve as a model for a highly proliferative hematopoietic cell population, can be transfected highly efficiently with our vector system. We demonstrate the generation of stable isogenic transgenic K562 cells which stably express dTomato without selection. These isogenic cells represent an important cell line for assays in the development of new immunotherapeutic approaches. K562 express a variety of different epitopes including CD33 which is an important target in the immunotherapy of acute myeloid leukemia. In contrast to K562, the transfection efficiency of primary human HSPCs was lower, with scope for improvement. However, our results demonstrate the ability of nS/MAR to transfect HSPCs even from poor-quality cryopreserved leukapheresis material. These findings provide a promising first step towards the genetic modification of HSPCs with nS/MAR and their usage in hematopoietic cell transplantation for the treatment of genetic hematological disorders. Further studies are ongoing to determine the optimal conditions for efficient modification of HSPCs including electroporation and expansion protocols.

870 Analysis of Cross-Reactivity of Anti-AAV Antibodies Elicited in Cynomolgus Monkeys Administered with AAV5, 8, or 9 Vectors

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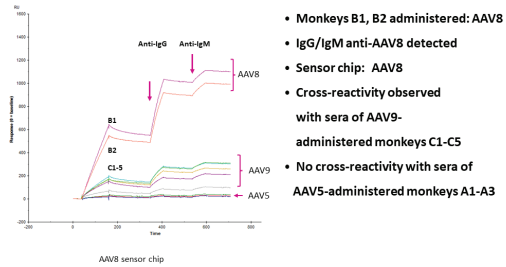
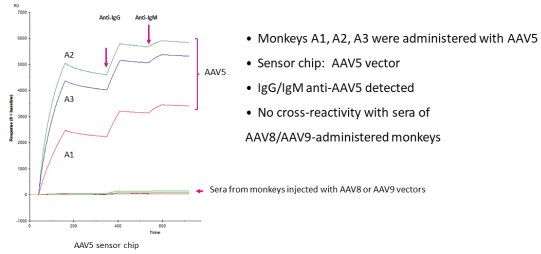
In the case of gene therapy delivered by adeno-associated virus (AAV) vectors, therapeutic protein expression may decrease over time. We were interested to test whether the intravenous administration of either AAV5, 8, or 9 in monkeys led to cross-reactive antibodies. The Surface Plasmon Resonance (SPR)-based immunoassay allows for label-free detection, automated operation, rapid analysis in real-time, and high specificity. We developed a Biacore SPR-based total binding antibody (TAb) assay for the direct detection of anti-AAV antibodies in the monkey serum to examine the cross-reactivity of anti-AAV antibodies after administration of AAV5, 8, or 9 vectors. AAV5, 8, and 9 vectors were immobilized onto a CM5 sensor chip surface on Fc2, Fc3, and Fc4, respectively by amine coupling. Fc1 served as a reference. Serum samples then flowed across 4 flow channels, followed by injection of anti-monkey IgG and IgM antibodies to determine isotypes. Cynomolgus monkeys were intravenously administered with AAV5, AAV8, or AAV9 vectors. To evaluate anti-AAV antibody responses after AAV administration, we analyzed neutralizing antibodies (NAb) and TAb against the AAV serotypes in the serum with cell-based NAb assay and Biacore-based TAb assay on 15- and 29 days after administration. While all 10 monkeys developed NAb against administered AAV capsids with a titer of 1:10,000, only 2 monkeys developed cross-reactive NAb against one of the other AAV serotypes with titers of 1:100 and 1:1000, respectively. TAb determined by Biocore-based assay were found to be cross-reactive between antibodies against AAV8 and AAV9, but there was little cross-reactivity between antibodies against AAV5 and AAV8/9 serotypes. Both IgG and IgM TAb on 15- and 29-days post-dosing were detected by Biacore, and antibody profiles determined by Biacore and ELISA platforms were comparable. Taken together, the Biacore-based assessment confirmed the lack of cross-reactivity of anti-AAV5 antibodies to AAV8, 9, or vice versa. The lack of cross-reactive antibodies against an AAV serotype may leave the possibility of re-administration of a different AAV serotype.

Cross-reactivity of anti-AAV9 antibodies against AAV8 vector on sensor chip by Biacore



Anti-AAV5 antibodies in monkeys injected with AAV5 vectors were detected by Biacore



871 A Screening Strategy of Synthetic AAVs for Cell Type-Specific Targeting and Precise Gene Therapy

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The ability to replace or edit genes has the potential to treat many genetic diseases. However, targeting specific cells remains a major challenge since few cell type-specific enhancers or promoters were identified. Here, we present a strategy for selecting adeno-associated virus (AAV) variants that express genes in specific types of tissue cells in vivo. By applying this strategy, we identified AAV variants transducing different types of cochlear cells, including inner and outer hair cells, supporting cells, outer pillar cells, inner border cells, and spiral ganglion neurons with superior efficiency and selectivity. We showed that specific AAV variants enabled precise gene therapy in the Tprn-/- mouse deafness model. Those cell type-specific AAVs would be valuable for functional studies and gene therapies for deafness, and the strategy could be potentially applied to screen specific AAV variants in other tissues.

872 Quantitative, Image-Based Assays for the Functional Evaluation of RNA and Gene Therapies Targeting Muscle Disorders

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Significant advances in RNA and gene therapies have finally enabled the clinical development of targeted therapies for muscle disorders. However, for many programs, clinical trials have been hampered by clinical holds. The lack of suitable cell-based functional assays that can be performed under physiological conditions and can serve as potency assays for product release is a critical challenge for these programs during development. We pioneered MyoScreen™, an *in vitro* R&D platform that enables testing muscle therapies in primary and immortalized patient-derived skeletal muscle cells. Under MyoScreen conditions, myoblasts are differentiated on micropatterned plates resulting in standardized and well-differentiated myotubes. Taking advantage of these properties, we have developed image-based, automated assays that enable the functional evaluation of RNA and gene therapies *in vitro*. The first group of assays monitors the colocalization of proteins within protein complexes that are critical for muscle functions, such as the interaction of dystrophin with other components of the Dystrophin-Glycoprotein Complex. A second group of assays uses Artificial Intelligence (AI)-powered cell profiling to define disease-associated cellular phenotypes in myotubes. Utilizing phenotypical profiles derived by machine learning from myotubes labeled for disease relevant proteins or cellular features, we define a Health-Score™ (% phenotypically healthy cells out of total) that quantifies the ability of therapies to revert disease-associated phenotypes. By imaging proteins that are part of the Dystrophin-Glycoprotein Complex, we demonstrate that these assays reproducibly and robustly monitor the function of dystrophin and assess the activity of restored dystrophin in DMD patients treated with gene and RNA therapies. The same assays can also be used to monitor therapies targeting DM1 where the recruitment of RNA splicing proteins by extended CUG repeats in the DMPK pre-mRNA leads to alternative splicing of dystrophin. Modifying the proteins or cellular features used for imaging enables the adaptation of these assays to other muscle disorders. In addition to their use as potency assays, these assays can predict the response of individual donors to therapies and can be applied for target and lead identification, to characterize mechanisms of action, and for the identification of biomarkers. Moreover, the phenotypical approach of the AI-powered cell profiling offers new opportunities for target and lead identification in neuro-muscular disorders with diverse or complex mechanisms.

873 Polymeric Nanoparticles for *In Utero* Gene Delivery in the Fetal Rhesus Monkey

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Introduction: Advances in fetal medicine have dramatically improved the ability to diagnose genetic diseases prenatally. These advances present opportunities to consider treating these disorders early during development. Fetal intervention provides a means to avoid pathology and tissue damage; gain access to privileged anatomical sites and unique stem and progenitor cell populations; and to administer a low dose-to-volume ratio for therapeutic efficacy. In small animal models, polymeric nanoparticles (NPs) have been demonstrated to effectively deliver gene-editing agents to target sites *in utero* [1]. For clinical translation, this technology must be shown to be safe and validated in a large animal such as the rhesus monkey. Here, we investigated biodistribution after polymeric NP delivery into the fetal rhesus monkey under ultrasound guidance. **Methods:** A block co-polymer of poly(amine-co-ester) (PACE) and polyethylene glycol (PEG) polymer was synthesized using enzyme-catalyzed copolymerization as previously described [2]. Poly(lactic-co-glycolic acid) (PLGA) and PACE-PEG NPs encapsulating lipophilic fluorescent DiD dye were formulated by a single-emulsion evaporation method. PACE-PEG NPs encapsulating mRNA for Green Fluorescent Protein (GFP) were formulated by a double-emulsion evaporation method. Time-mated rhesus monkeys were identified as pregnant sonographically then selected for the study. Rhesus fetuses in the early second trimester were transferred using an intraportal approach under ultrasound guidance (100 mg/kg). Fetal tissues were collected post-hysterotomy ~24 hours after DiD NP injection or ~48 hours after GFP NP administration for analysis including whole tissue fluorescent imaging and frozen sections of tissues using fluorescence microscopy. Images were processed in Fiji and the relative fluorescence was calculated using a custom routine coded with MATLAB [3]. **Results:** PLGA NPs were found primarily in the fetal liver, while the PACE-PEG NPs showed a wider tissue biodistribution. **Conclusions:** PLGA and PACE-PEG NPs were successfully and safely distributed after *in utero* delivery via the fetal portal vein. PACE-PEG NPs were found in a range of fetal tissues, which is consistent with the delivery profile seen previously in small animal models. This initial work suggests that polymeric NPs are potential candidates for efficient fetal delivery of therapeutic nucleic acids. Further studies are required to address the potential clinical translation of this innovative technology. **References:** 1. Ricciardi, A.S., et al., *In utero nanoparticle delivery for site-specific genome editing.* Nat Commun, 2018. **9**(1):2481. 2. Zhou, J.B., et al., *Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery.* Nat Mater, 2012. **11**(1):82-90. 3. Bracaglia, L.G., et al., *High-throughput quantitative microscopy-based half-life measurements of intravenously injected agents.* Proc Natl Acad Sci USA, 2020. **117**(7):3502-3508.

874 Predictive Potential of Single Deleterious Variations to Infer on AAV2 and AAV2-13 Capsids Viability

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Adeno associated viral (AAV) vectors represent the leading platform for gene therapy. The exponential growth of clinical trials using AAV reflects the enormous potential of this system in various therapeutic areas. One of the key advantages of AAV vectors is the high versatility due to the large number of wild-type and engineered capsid variants, each characterized by specific tissue tropism. Capsid engineering strategies mainly rely on the generation of library of millions of variants followed by selection steps allowing to retrieve the best candidates. One of the major issues related to this approach is the high frequency of non-viable capsids. The incomplete understanding of the consequence of amino acid variations in capsid stability still poses a major problem in capsid engineering. In this context, the study of wild-type capsids might help in the identification of the regions that mostly tolerate the mutations. In 2020, we screened a cohort of 1319 human liver tissues and isolated 59 new capsid variants equally distributed in two distinct subtypes: AAV2 and AAV2-13 hybrids (La Bella T et al. 2020). AAV vectors derived from these new capsids were characterized in terms of manufacturability and *in vitro* transduction efficiency. In total, 70% of the capsids were successfully produced and infectious in cells, whereas 17 variants were non-viable. AAV production was correlated to capsid mutations in order to identify common features in non-viable variants. We postulated the independency of the mutations and developed a prediction method based on publicly available data. In 2019, Ogden and colleagues characterized the complete AAV2 capsid fitness landscape of all single-codon substitutions, insertions and deletions across the VP (Ogden PJ et al. 2019). The fitness score of the corresponding single amino acid variations was associated to each mutation present in our variants. Mutations were categorized in common and unique based on their frequency in our series. No differences in fitness score of common mutations was found in viable and non-viable capsids, whereas unique mutations had a significantly decreased score in non-viable capsids. Unique mutations displayed a bi-modal distribution which allowed the definition of a threshold for deleterious mutations. Deleterious mutations identified in our series and in Ogden library shared common features related to their localization in the 3D structure of the capsid, they inversely correlated with accessibility and localization in hyper variable regions. Moreover, deleterious mutations occurring in buried residues were enriched in hydrophobic-to-hydrophilic variations. Non-viable capsids were characterized by the presence of one or multiple unique deleterious mutations. We experimentally validated the effect of the predicted deleterious mutations by assessing capsid production of reverse mutants which resulted viable. In addition, this approach was validated on other collections of mutated AAV from public data.

Our model was able to infer the fitness of variants with multiple amino acid variations by assuming the independence of the single mutations with a 94% of accuracy. This novel approach might be useful to guide the design of engineered capsids and might be adapted to other AAV serotypes relevant in gene therapy field.

875 Focused Ultrasound-Mediated Intranasal Delivery of AAV to Targeted Brain Regions with Minimal Systemic Exposure

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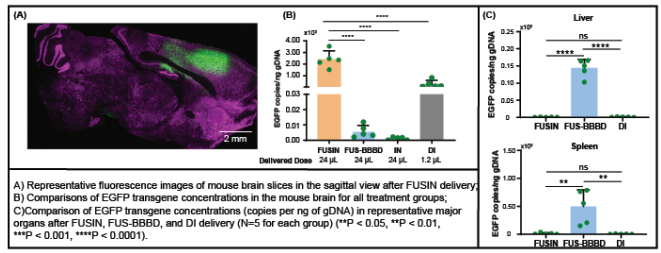
Background, Motivation and Objective Gene therapy is a promising approach for treating various brain diseases, and adeno-associated virus vectors (AAVs) have been extensively used as gene transfer vehicles to the central nervous system (CNS). Efficient and safe delivery of AAVs to the brain is the major challenge to the clinical translation of AAV-based gene therapy to treat brain diseases. Intravenous injection (IV) of AAV requires a unique BBB-permeable capsid design and suffers from the risks of severe systemic toxicity. Direct intracerebral injection (DI) of AAV is invasive, and the expression is limited to the injection region. This study aims to demonstrate that focused ultrasound-mediated intranasal delivery (FUSIN) can achieve safe and efficient delivery of AAV to spatially targeted brain locations with minimal systemic exposure in mice. **Statement of Contribution/Methods** AAV5 encoding enhanced green fluorescence protein (EGFP) fused to the Synapsin promoter (AAV5-hSyn-EGFP) was administered through the nasal route to the mouse brain, followed by focused ultrasound (FUS) sonication targeting the cortex in the presence of systemically injected microbubbles. Mice delivered with AAV5-hSyn-EGFP by DI, FUS-mediated blood-brain barrier disruption (FUS-BBBD), and intranasal delivery (IN) were used for comparison (n=5 per group). Four weeks following AAV delivery, mice were sacrificed and transcardially perfused. The mouse brains and other major organs were harvested for analysis, including histological staining of brain slices, and PCR quantification of EGFP transgene of mouse brains and major organs. **Results/Discussion** EGFP fluorescence signals were observed in the targeted cortical brain area, indicating that AAV5-hSyn-EGFP was successfully delivered by FUSIN and carried EGFP transgene was subsequently expressed (Figure A). The PCR quantification found that FUSIN achieved 414.9-fold higher EGFP transgene expression than FUS-BBBD ($p < 0.0001$), 2073.7-fold higher than IN ($p < 0.0001$), and 8.7-fold higher than DI ($p < 0.0001$), as shown in Figure B. Limited by the injection volume, the AAV dose for DI was 20.0-fold lower than that of FUSIN. FUSIN delivery resulted in minimal systemic accumulation in major organs, including the spleen, liver, kidney, and heart, when compared with FUS-BBBD and DI (Figure C). **Conclusion** The lack of noninvasive methods for efficient delivery of AAVs to the brain with minimal systemic toxicity limits the development of gene therapy for treating brain diseases. This study demonstrated that FUSIN achieved efficient AAV delivery and gene transduction at the targeted brain region with minimal systemic exposure of the AAVs to other major organs. Findings from this study suggest that FUSIN is a promising technology for AAV delivery to the brain with the potential to be translated to the clinic for the treatment of CNS diseases.



A) Representative fluorescence images of mouse brain slices in the sagittal view after FUSIN delivery; B) Comparisons of EGFP transgene concentrations in the mouse brain for all treatment groups; C)Comparison of EGFP transgene concentrations (copies per ng of gDNA) in representative major organs after FUSIN, FUS-BBBD, and DI delivery (N=5 for each group) (**P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

876 High Resolution Biodistribution Analysis Following Suprachoroidal Administration of a Pool of AAV3B, AAV8, and AAV9 Vectors to Non-Human Primates Reveals Spatial and Cell-Based Tropism Differences

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REGENXBIO Inc., Rockville, MD

Delivery of adeno-associated viral (AAV) vectors to the eye has the potential to address unmet needs associated with many ocular disorders. While subretinal (SR) delivery of AAV can be an effective therapy due to significant transduction of the retina and retinal pigment epithelium (RPE), it is a specialized procedure requiring surgery in an operating room. Intravitreal (IVT) delivery, while less invasive, has been associated with significant ocular inflammation. Targeted delivery to the suprachoroidal space (SCS) using transscleral microneedles can be performed in-office and may cause less inflammation than IVT delivery. AAV transduction following SCS delivery is not well-characterized compared to SR and IVT. In this study, we assessed biodistribution and transduction efficiency of a small pool of AAVs following SCS delivery in cynomolgus macaques. Based on the results of a pilot study in which we delivered a 118-member AAV library to the SCS in three NHPs, we selected AAV3B, AAV8, and AAV9 for further evaluation. AAVs were each produced twice, once packaging a primate protein-coding sequence with a unique barcode and once packaging a fluorescent reporter (GFP, tdTomato, or iRFP670). The six resulting preps were then formulated into a six-member pool, with fluorescent reporter preps comprising 85% of the vector pool and native transgene preps comprising 15%. This formulation method allowed us to obtain comparable biodistribution and expression data from each capsid by PCR and next-generation sequencing (NGS) using the vector genomes and mRNA transcripts from the primate protein-coding gene constructs as well as to visualize protein expression of each fluorescent reporter by histology. Following suprachoroidal delivery of the resulting pool to both eyes of three NHPs, right eyes were fixed whole for histology and left eye tissues were harvested for PCR/NGS three weeks later. Posterior tissue was dissected into quadrants, and 6mm punches from central and peripheral regions of the retina, RPE/choroid, and sclera from each quadrant were collected. Irrespective of location, retina, RPE/choroid, and sclera contained 0.5, 3.8, and 95 GC/cell on average resulting in 0.12, 3.3, and 44 transgene RNA copies/TBP mRNA copy, respectively. These data suggest that retinal and RPE/

choroid transduction by AAV is less efficient overall than scleral. After spatially disaggregating the data, peripheral regions were more highly transduced than central regions in all tissues: 2.7 to 14-fold by DNA and 5 to 40-fold by RNA. By NGS analysis, AAV3B produced 1.7-fold more RNA in retina than AAV8 with equivalent DNA abundance. AAV8 produced 1.5-fold more RNA in RPE/choroid than AAV3B; however, after normalization by DNA abundance, AAV8 produced 10-fold less RNA per vector genome than AAV3B. Immunofluorescence revealed that both AAV3B (expressing tdTomato) and AAV8 (expressing GFP) preferentially transduced the RPE over the choroid, transducing 5-30% of cells in regions analyzed. NGS analysis of the sclera revealed that AAV3B transduced sclera 10-fold less efficiently than AAV8. Scleral expression of tdTomato was detected in less than 5% of the area of scleral regions of interest (ROIs), compared to up to 17% of the same scleral ROIs being GFP+. By NGS, AAV9 transduced RPE similarly to AAV3B, retina similarly to AAV8, with a modest 2-fold decrease in scleral transduction compared to AAV8; histological analysis is ongoing. Taken together, these data suggest that AAV3B and AAV8 have discrete tropisms following delivery to the SCS in NHP and their use may have unique advantages in an ocular gene therapy setting.

877 High-Throughput Method to Analyze the Cytotoxicity of CAR T Cells in a 3D Tumor Spheroid Model Using Image Cytometry

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Chimeric antigen receptor (CAR)-T cell therapy is an antigen-dependent cellular therapy that has gained considerable traction in the field of cancer immunotherapy. CAR-T cell therapy involves specifically engineering T cells to attack tumor cells by binding a tumor antigen and inducing T cell activation resulting in intracellular signaling and cytokine release. Currently, there are six FDA-approved CAR-T cell therapies, which all target the CD19 or BCMA antigens for hematologic B cell malignancies. In recent years, a strong focus has been placed on CAR T cell therapy discovery for solid tumors, which may better recapitulate physiological conditions, thereby potentially improving the selection of CAR construct candidates. Immune cell trafficking and immunosuppressive factors increase the relative difficulty in developing a robust CAR-T cell therapy against solid tumors. Therefore, it may be critical to develop novel methodologies for high-throughput phenotypic and functional assays using 3D tumor spheroid models to better assess CAR-T cell therapies against solid tumors. Recently, plate-based image cytometry has emerged as a method to investigate and characterize CAR T cell functions in a high-throughput manner. Image cytometry has demonstrated capabilities in analyzing transduction efficiency, cell proliferation, and cytotoxicity for CAR T cell therapy. With the development of 3D spheroid models, image cytometry may provide the necessary tools and applications for CAR T cell therapy discovery geared towards solid tumors. In this work, we discuss the use of CAR-T cells targeted towards PSMA, an antigen that is found on prostate cancer tumor cells, the second most common cause of cancer deaths among men worldwide. Herein, we demonstrate the use of high-throughput plate-based image cytometry to characterize PSMA CAR-T cell-mediated cytotoxic potency against 3D prostate tumor spheroids and simultaneously monitor location of the T cells *in vitro*. We were able to kinetically evaluate the efficacy and therapeutic value of PSMA CAR-T cells by analyzing the cytotoxicity against prostate tumor spheroids. Furthermore, the T cells are fluorescently labeled with a tracer dye to visually locate the cells on the tumor spheroids. The proposed image cytometry method can potentially overcome limitations placed on traditional methodologies to effectively assess cell-mediated 3D tumor spheroid cytotoxicity and efficiently generate time- and dose-dependent results.

878 ATHENA I System: A High-Throughput AAV Variant Comparison and Selection Platform

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AAV vectors are powerful tools for gene delivery and therapy, however, currently used AAVs are of low efficiency, which leads to high doses, high side effects, high costs and also manufacturing problems. Hundreds of identified natural AAV serotypes and an increasing number of engineered variants allow us to find highly efficient AAVs for each cell type and tissue, although it is difficult to identify the best AAV for each target quickly. To solve this problem, we have developed a novel platform, ATHENA I, for rational capsid comparison and selection. In this platform, each capsid variant packages an identical reporter gene with three unique DNA barcodes. Hundreds of AAV vectors can be generated at once, pooled and tested together in vivo or in vitro. DNA and transcribed RNA barcodes are then quantified by NGS to evaluate putative capsids for each target. Efficacy of each AAV candidate is obtained by directly comparing the abundance of the barcodes in NGS data before and after infection rather than comparing with other AAV variants. In this way, it reduces most of the variables and the results are more accurate and reliable. We harnessed the ATHENA I platform to evaluate 88 different AAV variants in two different mouse models after systemic injection: wt 129/SV and liver-humanized C57BL/6. At RNA level, AAV8-Y447F-Y733F, AAV-DJ, Anc80L65, AAV8, AAV10.1, AAV-DJ8, AAV-Rh.74, AAV10, AAV-SCH9 and AAV7 were the top 10 AAV variants in wt 129/SV liver. At DNA level, AAV-DJ, AAV2-Y444F-Y500F, AAV-Rh.74, AAVrh10, AAV10.1, AAV7, AAV8, AAV-DJ8, AAV7-Y446F-Y732F and AAV-SCH9 were the top 10 AAV variants. The performance of each AAV variant was different in liver-humanized C57BL/6. The top 10 AAV variants at RNA level were AAV9-PHP.S-Y354H, AAV9-PHP.S, AAV9-F, AAV9-Y446F-Y731F, AAV6.2FF, AAV-DJ, AAV8-Y447F-Y733F, Anc80L65, AAV-SHH10 and AAV9-PHP.B and the top 10 at DNA level were AAV2-retro, AAV9-Y446F-Y731F, AAV9, AAV3B-Y445F-Y731F, Anc80L65, AAV2, AAV9-PHP.S, AAV9-PHP.S-Y354H, AAV-DJ8 and AAV3B. These results confirmed that AAV8 and its Y/F mutants are better candidates for mouse liver, while in the liver-humanized C57BL/6, AAV9 and its variants outperform both at RNA and DNA levels. With ATHENA I, we also confirmed AAV9-PHP.eB and AAV9-PHP.B had the best performance to cross blood-brain barrier (BBB) in both RNA and DNA levels in the brain of liver-humanized C57BL/6, but they didn't work very well in wt 129/SV mouse brain. In contrast, AAV-F had the highest RNA and DNA in 129/SV mouse brain. AAV6.2FF, AAV10, AAV6.2, AAV5 and AAV-Rh.74 showed similar or higher crossing BBB activity compared with AAV9

in wt 129/SV mouse as measured by RNA expression. At DNA level, AAVrh10, AAV-Rh.74, AAV9-Y446F-Y731F, AAV7, AAV-DJ, AAV7-Y446F-Y732F, AAV-DJ8, AAV2, and AAV2-Y444F-Y500F were better than AAV9. In the liver-humanized C57BL/6 moue brain, AAV6.2FF, AAV10.1, AAV1, AAV4-Y729F, AAV1-Y445F-Y731F, and AAV10 had better performance at RNA level, while AAV1-Y445F-Y731F, AAV-Rh.74, AAV1, AAVrh10, AAV-DJ8, AAV7, AAV10.1, AAV9-Y446F-Y731F, AAV8, and AAV7-Y446F-Y732F had better performance at DNA level compared with AAV9. These results showed that rather than AAV9, other AAV serotypes could be used as template for AAV capsid library construction to select the AAV variants which are able to cross BBB. These results showed AAV variants in a different models, different detection level(DNA and RNA) will have different outcomes. A cross-species evaluation may help select potent AAV variants for human gene therapies. With ATHENA I, we can effectively compare efficiency of hundreds of AAVs in a fast and high-throughput way in different animal models in both DNA and RNA levels, select the best AAV for a specific disease therapy, and design even better AAVs. We now have more than 1000 AAV variants and similar works have been executed in NHPs. The results will be reported soon. Multiple tissue-specific ATHENA I kits are also available for collaboration.

879 Enrichment of Genetically Modified Hematopoietic Stem Cells through Multiplex Base Editing

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β-hemoglobinopathies are caused by mutations affecting the adult hemoglobin (Hb) production. Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing hereditary persistence of fetal Hb (HbF) (HPFH) mapping to the fetal γ-globin (*HBG*) gene promoters. Base editing (BE) allows the introduction of HPFH point mutations (C>T by cytidine base editors, CBEs; A>G by adenine base editors or ABEs) without generating double-strand breaks (DSBs).We previously reported that introduction of HPFH mutations in the *HBG* promoters using CBE or ABE leads to therapeutically relevant HbF levels in red cells differentiated from patient's HSPCs. Xenotransplantation experiments showed BE in long-term HSCs; however, the efficiency was reduced compared to input HSPCs. BEs might have induced some toxicity in *bona fide* HSCs or might be less efficient in this cell population compared to progenitors. *Ex vivo* or *in vivo* enrichment of edited HSCs could be exploited to maximize the engraftment of genetically modified cells. This could also allow the reduction of the conditioning regimen and its side effects. Such an approach could increase the success of therapeutic strategies for diseases with no or little selective advantage of corrected cells such as β-hemoglobinopathies.Here, we developed a strategy enriching for base-edited HSPCs to achieve high BE efficiencies *in vivo*, and eliminate unedited HSCs that might have a survival advantage and better stemness compared to edited cells. We used BEs (that allow multiplex editing without generating genomic rearrangements) to simultaneously target the *HBG* promoters and insert HPFH mutations, and the *CD33* gene to downregulate CD33, a receptor expressed by HSCs with a high regenerative potential, but dispensable for hematopoietic cell activity. Selection of *CD33* knock-out (KO) HSPCs will also enrich for cells edited at the *HBG* promoters.We designed sgRNAs that target *CD33* and, when coupled with CBEs or ABEs, disrupt *CD33* start codon, or splice donor/acceptor sites. We screened sgRNAs in an erythroid cell line (K562) and identified efficient sgRNAs downregulating *CD33*, achieving BE efficiencies of up to 80%, and a concomitant 5-fold CD33 downregulation. We then performed multiplex base editing by simultaneously targeting *HBG* and *CD33* in K562 cells. We observed no statistically significant difference in BE efficiency between single and dual editing conditions. Upon FACS-sorting based on CD33 expression, we observed higher BE efficiencies at *HBG* in $CD33^-$ vs $CD33^+$ populations, thus indicating elimination of poorly edited cells in the $CD33^-$ compartment.We applied the dual editing strategy in patient HSPCs selected based on the expression of CD34 (a typical marker of HSPCs). Two days post-transfection, control and edited cells expressed similar CD34 levels, while only edited cells showed a strong decrease in CD33 expression. $CD33^-$ and $CD33^+$ populations were FACS-sorted. Importantly, we detected higher BE efficiencies in $CD33^-$ *vs* $CD33^+$ populations at *HBG* promoters. A colony-forming cell assay revealed no difference in the progenitors' number, differentiation and viability between control and $CD33^-$ sorted populations.In conclusion, we showed that multiplex editing of *HBG* promoters and *CD33*, in conjunction with $CD33^-$ selection leads to high BE efficiencies and eliminates poorly edited cells. Importantly, this strategy can be used to enrich for edited cells and develop effective therapeutic for many hematopoietic and non-hematopoietic diseases amenable to HSC-based gene therapy treatment.

880 Development of Oral Cancer Vaccine by Using Recombinant Saccharomyces Cerevisiae Displaying Wilms' Tumor 1 Protein

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In recent years, cancer immunotherapy using immune checkpoint inhibitors has been used with great success in treatment of various types of cancer such as bladder cancer. However, monotherapy of immune checkpoint inhibitor by using anti-programmed death-1 (PD-1) showed the objective response rate of approximately 21% in advanced bladder cancer. Therefore, combination of immunotherapy will be a promising modality to improve the response in bladder cancer patients. Previously, we had constructed a recombinant *Bifidobacterium longum* displaying Wilms' tumor 1 (WT1) protein, and had shown the significant anti-tumor effect in mice. In this study, we newly constructed recombinant *Saccharomyces cerevisiae* displaying WT1 protein (BY-WT1) to achieve more efficient expression of the WT1 protein, higher productivity and higher efficiency in anti-cancer effect than predecessor. To construct the BY-WT1, parental *S. cerevisiae* strain was transformed by plasmid vector and the protein expression was determined by real-time RT-PCR and western blotting. To investigate the anti-tumor effect of BY-WT1, 3.6 mg per dose of BY-WT1 was orally administered 5 times a week into C3H/He mice bearing syngeneic MBT-2, a murine bladder cell line. Intraperitoneal

injection of 10 μg of anti-PD-1 antibody was performed with oral administration. Spleen cells were isolated to determine WT1-specific T cell immune responses and cytotoxicity against MBT-2 *in vitro*. As the results, recombinant *S. cerevisiae* successfully expressed WT1 protein on the yeast cell wall. Oral administrations of BY-WT1 remarkably inhibited the tumor growth as well as anti-PD-1 therapy in mice bearing MBT-2. In addition, combination therapy of BY-WT1 and anti-PD-1 significantly decreased the tumor growth compared with PBS control at days 29 after tumor inoculation ($p<0.05$). Oral administrations of BY-WT1 significantly increased the population of CD4T and CD8T cells that were secreting WT1-epitope-specific cytokines such as IL-2, and TNF-α in mice ($p<0.05$, respectively). Furthermore, oral administration of BY-WT1 induced remarkably higher cell cytotoxic activity in splenocytes against MBT-2 *in vitro*. In conclusion, we showed that the combination of oral administration of recombinant *S. cerevisiae* displaying WT1 and intraperitoneal injection of immune checkpoint inhibitor induced WT1-specific T cell responses and resulting anti-tumor effect in syngeneic mice model. Our findings suggested that the cell surface engineering technology for *S. cerevisiae* could be a possible approach for intestinal immune system and BY-WT1 may be a new therapeutic candidate for advanced bladder cancer.

881 An Adeno-Associated Virus-Based Gene Replacement Therapy for Type II Gaucher Disease

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Gaucher disease (GD) is a rare genetic lysosomal storage disorder caused by mutations in the glucosylceramidase beta 1 (GBA1) gene which encodes lysosomal enzyme acid GCase. Inadequate activity of GCase causes the progressive accumulation of the sphingo-lipid glucosylceramide in lysosomes of macrophages (referred to as Gaucher cells), mainly in the liver, spleen, bone, and bone marrow, which eventually leads to the dysfunction of multiple organs. There are three types of GD, Types 1, 2, and 3). GD2 and GD3 are less common than GD1 and are mainly dominated by neurologic pathologies in terms of clinical presentation, especially GD2. The ERT of GCase protein has been developed for treating GD1 to ameliorate most of the symptoms and improve patient's quality of life. For GD2 patients, there is no effective treatment since the recombinant enzyme drug cannot pass through the blood-brain barrier and has limited effects on reducing neurological symptoms. In GD3, the progression of brain damage is not as rapid as GD2, so ERT can relieve most of the systemic manifestations and extend patients' life-span significantly, though a retarded mental development (encephalopathy) is often observed. So, new treatment is urgently needed to restore the GCase activity in the central nervous system (CNS), to help patients that are diagnosed with GD2 and GD3. We have developed an investigational gene therapy, VGN-R08b, which utilized non-replicating virus (a recombinant AAV9) as a vector to deliver genetically modified functional human GBA1 gene into human cells. By intra-cerebroventricular administration of VGN-R08b, we aim to restore the GCase activity in the brain of GD2 and GD3 patients, thus relieving the neuropathic symptoms caused by the accumulations of sphingo-lipid glucosylceramide in the CNS with a one-time treatment. Our results showed that in a GCase loss-of-function cellular model, VGN-R08b effectively restored the GCase activity. In a conduritol B epoxide (CBE) induced mouse model of GD2, VGN-R08b significantly improved the viability, locomotor activity, and motor coordination of the GD-induced mice. Preliminary mouse safety studies demonstrated that VGN-R08b is well tolerated. An investigator-initiated-trial of VGN-R08b is ongoing in China.

882 Human RHO Editing for Treatment of Autosomal Dominant Retinitis Pigmentosa

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Retinitis Pigmentosa (RP) affects 1/3.000 patients worldwide, with 30-40% of cases having an autosomal dominant (AD) inheritance. The rhodopsin gene (RHO) is the most commonly mutated in AD RP patients (RP4), with the P23H gain-of-function mutation being the most common in US. Canonical gene therapy approaches that add a correct copy of the gene, are not beneficial and removing the mutant RHO allele is necessary to avoid its toxic effect. The CRISPR-Cas9 based allele-specific editing has emerged as a therapeutic option for dominant RP by blocking the production of the toxic protein without affecting the correct copy of the gene. However, this approach limits its applicability to all RP4 patients, as design of a gRNA specific for each mutation would be required. We have recently described Homology-Independent Targeted Integration (HITI) which uses adeno-associated viral (AAV) vectors to deliver both CRISPR-Cas9 and a donor template to be integrated at the mouse RHO locus. This blocks the expression of the endogenous RHO and replaces it with its wild type copy. Here we have designed AAV-HITI that targets the human RHO locus, and tested it in a recently described P23H knock-in mouse model of RP4 .We performed subretinal injections into hRHO-P23H-tagRFP mice, with two AAV8 vectors, encoding the Cas9 and the donor DNA (carrying both RHO and GFP to label photoreceptors where integration occurred) respectively. The contralateral eye served as control. HITI efficiency was calculated by microscopy analysis on retinal sections by counting the GFP fluorescence in the outer nuclear layer (ONL).One month after injection, retinal electrical activity was measured by electroretinogram (ERG) in homozygous mice.At 30 days post-injection, we have observed HITI efficiency up to 12±8% in the transduced area (n=4) and an improved retinal electrical function (B-wave) as measured by ERG (p=0,005; n=10). Prospectively, we plan to evaluate the improvement in the retinal phenotype at advanced timepoints and potential off-targets editing events. Our preliminary results provide the first proof-of-concept of the efficacy of HITI as a therapeutic strategy for AD RP due to RHO mutations in a humanised mouse model and therefore, it could be readily translated to a human setting.

883 Human Beta2-Microglobulin (B2M) Knockdown as an *In Vitro* Model to Evaluate CRISPR Delivery

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Previously, our lab reported gold nanoparticles carrying CRISPR components (CRISPR-AuNP) for gene editing primary human hematopoietic stem and progenitor (HSPCs) ex vivo. Optimization of CRISPR-AuNP include changes to the target locus for editing, homology directed repair templates, addition of other moieties for altered function and specificity, and changes to steps in formulation. With each alteration, testing efficiency of gene editing and toxicity in vitro can help determine potential translation to animal models. However, DNA sequencing alone takes time and resources, and isn't always relatable to functional changes in cellular biology at the protein level. We sought to establish a high-throughput, reliable in vitro model for evaluating multiple delivery strategies across human cell and tissue types. The human β-2-microglobulin (B2M) gene is a small, highly conserved sequence whose protein product is a component of major histocompatibility complex (MHC) class I, expressed ubiquitously on the surface of most cell types. Other groups have documented the utility of CRISPR-based editing of B2M for therapeutic purposes in immunology, with indels resulting in loss of B2M surface protein expression measured by flow cytometry. Here we tested B2M as a single-gene target model for CRISPR delivery in vitro. Readouts included loss of cell surface B2M protein expression, MiSeq™ next generation DNA sequencing, and T7 endonuclease I (T7EI) assay. We tested Jurkat and K562 cells by electroporating Cas9 or Cas12a ribonucleoprotein (RNP) complexes targeting B2M but observed low protein knockdown with the published Cas12a guide RNA sequences (gRNA) (64.4%±2.3% for Cas9 and 2.9%±1.1% for Cas12a). We identified five additional Cas9 gRNA binding sites and five Cas12a gRNA binding sites to test. Electroporation studies in HSPC (n = 3 unique donors) and primary human T cells (n = 1 unique donor) identified one gRNA for each nuclease type which resulted in superior gene editing by flow cytometry, MiSeq™ and T7E1 (Fig. 1a and 1b, respectively). We tested this approach in other cell lines by electroporating RNP with or without interferon gamma (IFN-γ) to increase B2M expression and compared to mock-treated samples as a negative control. Cells were analyzed 48-hours post electroporation. All seven cell lines tested showed > 5% reduction in B2M surface protein expression compared to control with either a Cas9 or Cas12a gRNA (Fig. 1c, range: 5%-58%), which is currently being validated by MiSeq™ and T7EI assay. Finally, we tested CRISPR-AuNP delivery, originally validated at a different, non-B2M genetic loci, with an updated particle design (See abstract [2023-A-1421_ASGCT]) in HSPCs (n=1 unique donor), which resulted in 16.5%±4.6% B2M protein knockdown by flow cytometry, 8.3%±3.7% editing by MiSeq™, and 2.6%±1.8% editing by T7E1 (Fig. 1d). This suggests that CRISPR-AuNP delivery is associated with increased gene editing readout by flow cytometry compared to DNA sequence analyses. We later determined this to be a function of increased false positive signal from cationic polymer components (See abstract [2023-A-1795_ASGCT]). Altogether this study suggests B2M is a useful model for comparing gene editing efficiencies in vitro as a function of method of delivery.

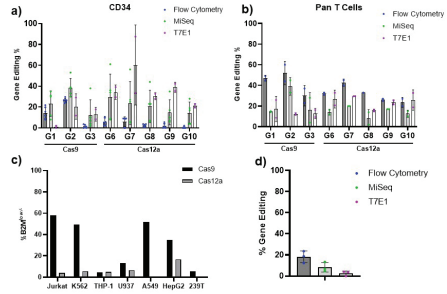

Figure 1. Evaluation of CRISPR gRNAs targeting B2M in a) G-CSF mobilized CD34+ cells (n=3 unique donors) b) Pan T cells (n=1 unique donor) c) immortalized cell lines (n=1) as follows: Jurkat (T cells); K562 (Bone; Marrow); THP-1 (Monocytes); U937 (Macrophages); A549 (Lung); HEPG2 (Liver); 293T (Kidney). d) CRISPR/Cas9-AuNP administered to HSPCs (n=1 unique donor).

884 Cardiomyocyte Death after AAV Gene Transfer Can Be Related to Proteotoxicity or to Transgene Abnormal Function or Localization

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Gene delivery technologies based on adeno-associated virus vectors (AAV) have yielded promising results for the treatment of monogenic diseases, leading in recent cases to marketing authorization in Europe and US. However, preclinical studies and clinical trials have highlighted that infusing high vector doses can trigger immune responses or induce cytotoxicity in a number of organs, leading in a few cases to the death of the patient. In the wake of signs of cardiac suffering in humans after gene transfer in SMA, DMD and MTM1, we investigated the consequences on the heart of administration of high doses of AAV vectors expressing 3 different transgenes corresponding to muscular dystrophies: a form of microdystrophin for Duchenne Muscular Dystrophy, the human full-length SGCG cDNA for γ-sarcoglycanopathies and the human full-length FKRP cDNA for FKRP deficiencies. We observed in all these cases the possibility of cardiotoxicity with high doses injection. Preventing transgene expression in heart through the use of a cardiac specific microRNA, miR208a, was shown to prevent the appearance of such cardiotoxicity, indicating that the cardiotoxicity was related to transgene expression in this organ. Dissection of the mechanisms at stake revealed activation of different cascades leading all to cardiomyocyte death. Overall, our data highlighted a specific sensitivity of the heart to transgene expression, in particular compared to skeletal muscle, suggesting the importance to regulate finely the expression of transgene in this organ in any gene therapy approach. The manuscript presenting this work is ready for submission.

885 Using TcBuster™ (TcB-M™) Transposase for Highly Efficient and Robust Delivery of Multicistronic Therapeutic Cargo in Immune Cells for Both RUO and Clinical Applications

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Rapid development of genome engineering tools has driven several immune- and stem cell therapies in clinical trials with the goal of generating autologous and allogeneic therapeutics. Many of these therapies use viral vectors for the delivery of therapeutic cargo. However, viral mediated therapies carry the risk of immunogenicity, cargo size limitations, integration site risk, manufacturing delays, and is highly cost prohibitive. While there are a couple of known non-viral transposase-based systems, such as, PiggyBac and Sleeping Beauty, both are exclusively licensed and not available for commercial use. TcBuster-M™ (TcB-M™) is a commercially available non-viral transposase-based editing platform that overcomes current viral limitations. TcBuster is found in the red flour beetle and is a member of the hAT family of transposases. Using direct evolution, we engineered a hyperactive mutant (TcB-M) that has improved transposition rates using less mRNA transposase and plasmid DNA transposon. Divergent from the engineering efforts used to build hyperactive enzymes of PiggyBac and Sleeping Beauty, we used a novel high-throughput screening platform in mammalian cells. This allowed us to screen a mutant library of > 3 million variants, which is much larger than those used to build PiggyBac or Sleeping Beauty. This led to the construction of the most efficient transposase system for engineering primary immune cells. TcB-M allows for rapid cell manufacturing with limited cell manufacturing cost. Current TcB-M timeline from vector map to GMP transposon is ~6-8 months. Since TcB-M is less constrained by cargo size, we can design large multicistronic transposons for robust delivery of multiple proteins in various cell types, including primary T- and NK- cells, mesenchymal stem cells, and induced pluripotent stem cells (iPSCs). Additionally, TcB-M can be easily combined with endonucleases, such as CRISPR reagents, to generate combinatorial knock-out/overexpression edited cell products. The improved TcB-M has resulted in cargo integration rates of over 60% in primary T-cells and peripheral blood derived NK cells, without sacrificing cell growth or clonal dominance concerns. Finally, we have conducted direct comparisons against lentiviral, PiggyBac, and Sleeping Beauty engineered CAR-Ts, demonstrating TcB-M engineered CAR-Ts has equal to higher integration percentage. TcB-M also has a safer integration profile as it is more randomly integrated into the genome without preference to active sites when compared to Lentivirus. Overall, TcB-M is a widely available proven non-viral gene editing technology to deliver large or difficult therapeutic cargos in a variety of cell types. TcB-M thus reduces many of the viral mediated editing hurdles, allowing faster generation of crucial therapeutics to market.

886 Development of an iPSC-Derived Cell Therapy for Limb-Girdle Muscular Dystrophy Type 2A/R1

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Limb-girdle muscular dystrophy type 2A/R1 (LGMD2A/R1) is a rare form of muscular dystrophy with around 4000 patients in the US and approximately 80-100 new patients identified every year. LGMD2A/R1 is caused by mutations in the CAPN3 gene, which leads to loss of functional calpain-3 protein, resulting in symmetrical muscle wasting of the proximal limb and girdle muscles. There is currently no approved treatment for LGMD2A/R1. Regenerative therapies derived from induced pluripotent stem cells (iPSCs) may provide a promising approach to regenerate muscle mass associated with muscle wasting. Here we describe the pre-clinical development of an iPSC-based autologous therapy for LGMD2A/R1. A CRISPR-based knock-in strategy was developed to replace the endogenous defective *CAPN3* gene with a functional version in LGMD2A/R1 patient-derived iPSCs. Using a chemically defined, step-wise myogenic lineage specification protocol, PAX7-expressing myogenic progenitor cells (MPCs) were then generated; upon myogenic fusion induction, these cells formed functional myotubes that produced *CAPN3* mRNA and protein. We then transplanted *CAPN3* edited LGMD2A/R1 iPSC derived MPCs into the injured TA muscles of NSG mice and demonstrated donor cell-derived muscle regeneration. Furthermore, a subpopulation of transplanted MPCs seeded in the local muscle stem cell niche area under the basal lamina in mice where they adopt a quiescent state and contribute to regeneration upon additional injury, thus providing evidence of a long-term maintenance and regenerative capability. To facilitate *in vivo* efficacy studies in an animal model of LGMD2A/R1, we generated an immunodeficient calpain-3 null mouse by back-crossing CAPN3KO mice (JAX: CC041-Capn3em10Lutzy/J) with NSG (JAX: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice. Intramuscular transplantation of *CAPN3* edited MPCs into injured muscle of CAPN3KO-NSG mice leads to donor-derived muscle fiber regeneration, supporting the rationale for continued development. Manufacturing of LGMD2A/R1 iPSC-derived MPCs to support IND-studies and first-in-human trials is ongoing with the goal of IND-filing.

887 Automated Micro-TFF System Streamlines Purification and Operator Time in a Lean rAAV Manufacturing Operation

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Recombinant Adeno-associated virus (rAAV) doses greater than or equal to 1E13 vg/kg are required for pre-clinical or clinical studies with increasing frequency. Producing the desired doses often requires concentrating rAAV drug substance from a starting volume of one hundred milliliters down to ≤ fifteen milliliters during final formulation. For these applications, Molecular Weight Cut Off (MWCO) centrifugal filtration devices (spin filters) are commonly

employed to concentrate and exchange buffers on the rAAV drug substance. MWCO spin filters can fulfill this function, but require long hands-on time for full buffer exchange or viscous starting products such as those containing iodixanol. Additionally, MWCO spin filters provide inconsistent process control due to dependencies on multiple variables (including volume, concentration, viscosity, and spin time) and lack in-spin progress monitoring. In a lean rAAV manufacturing operation, the operator hands-on time requirement represents a substantial production bottleneck. To relieve this bottleneck, we evaluated a commercially available small volume tangential flow filtration (TFF) device, which has walk-away automation and the ability to concentrate material from one hundred milliliters to one milliliter. In our evaluation, transitioning to an automated TFF system yielded advantages in operator hands-on time, step-yield, and process consistency. We additionally observed consistent performance across a variety of rAAV serotypes, thus demonstrating its value in a lean and serotype-varied rAAV manufacturing operation.

888 A Process Development Approach to a Platformable and Robust Scale Up for Enrichment of Full AAV Capsids Using Membrane Chromatography

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Adeno-Associated Virus (AAV) vectors are one of the current leading platform for gene delivery treatments. One of the most challenging aspects in manufacturing AAV is the removal of 'empty' capsids, which do not contain the gene of interest. Here we showcase a comprehensive approach for developing a robust scale-up process for the purification of full AAV capsids using Mustang® Q anion exchange membrane chromatography capsules. We have extended this approach to AAV serotypes 5, 8 and 9. Initial process development was performed using our novel 1 mS/cm conductivity step elution method. This multi-step elution strategy is a tool for screening for improved separation conditions and serves as a guide for conditions to facilitate elution of empty and full capsids with just two conductivity steps. We show a thorough two conductivity step, design space investigation, identifying operating conditions that achieve high purity and high yield. The selected operating space is then used to scale up this method into radial flow capsules such as the Mustang Q XT capsule 50 mL device. We present the results from our scale-up purification using Mustang Q XT capsules 50 mL and show purity and yield are comparable with the results obtained at the process development scale. Additionally, we show consistent performance across different Mustang Q capsules with several feedstreams from different industry relevant sources. Taken together, the data shows that Mustang Q capsules can be a robust and platformable process for the removal of empty AAV capsids.

889 A Computational Framework to Detect ITR Deletion for rAAV Vectors

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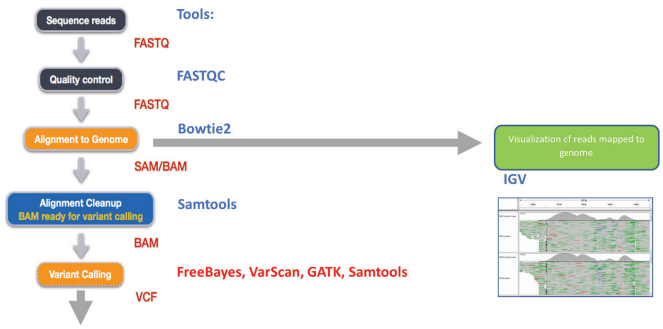
Adeno-associated virus (AAV) is a promising vector for gene therapy. However, manufacturing recombinant AAV (rAAV) vectors can be challenging. The inverted terminal repeat (ITR) sequences that flank the gene of interest form highly stable T-shaped hairpins, which are critical for replication and encapsidation of viral DNA. The palindromic nature and high GC content make ITRs prone to truncations. In addition, mutations are challenging to detect with NGS-based methods. Deletions in the ITR region can lead to reduced efficiency in viral packaging and increased variability in downstream experiments. Since there is a lack of bioinformatics tools for ITR-specialized detection, we established a robust computational framework to assess ITR deletions for rAAV vectors using NGS data. We start by running FastQC for quality control of sequencing data. We validate the quality of sequencing data for downstream analysis based on the per base sequence quality and per sequence quality scores from FastQC. Then we align the NGS reads to rAAV vector genome using Bowtie2 and visualize sequence reads mapped to the genome using Integrative Genomics Viewer (IGV). From the IGV visualization, we were able to hone into locations which genome regions have deletions as well as distribution of depth of coverage of sequence reads. To get consistent quantitative detection of deletions, we applied 4 standard variant calling tools (FreeBayes, VarScan, GATK, Samtools) at customizing input QC parameters to assess presence and significance of any observed deletions and mutations.As proof-of-concept, we applied this computational framework to detect ITR deletions in a batch of 8 rAAV vectors encoding the same genome. One variant calling method (VarScan) identified statistically significant 44 nucleotide (nt) deletions in 3' ITR, promoter, and CDS at a low frequency (<5%) in a subset of these vectors. We also establish the need to triangulate analyses from multiple variant calling tools to conclusively confirm deletions in the ITR sequences. Identifying mutations in complex genomic regions can save substantial costs down the line and lead to more reproducible results in manufacturing AAV gene therapies.



Output: Deletions and insertions called with frequencies and p-values assigned

890 Dose Response of Dual Route *scAAV9-HEXM* Gene Transfer in a Mouse Model of Sandhoff Disease

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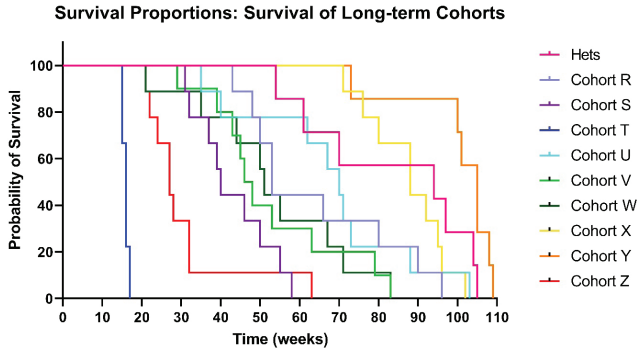
Sandhoff disease (SD) is caused by the excessive accumulation of GM2 gangliosides in the lysosomes of neuronal cells. Typically, these lipids are hydrolyzed by an enzyme, β-hexosaminidase A (Hex-A), a heterodimer comprised of an α- and a β-subunit. Mutations in the gene encoding either subunit can lead to improper functioning of the enzyme. SD is caused by a mutation in the *HEXB* gene resulting in a deficient or absent β-subunit and subsequent accumulation of GM2 gangliosides. This causes widespread cell death, and consequently progressive symptoms and rapid neurological decline culminating in death by age 4 in the most prevalent, infantile form of the disease. A homodimer formed by a novel hybrid μ-subunit called HexM, an isoenzyme of human Hex-A, has been recently developed and shown to hydrolyze GM2 gangliosides *in vivo*. Previous studies have determined the effectiveness of gene transfer with the gene, *HEXM,* packaged in a self-complementary adeno-associated viral vector, serotype 9 (scAAV9), through increased life span in a SD mouse model (*Hexb*$^{(-/-)}$). This study aims to determine the dose response of the *scAAV9-HEXM* treatment in the SD mouse model through dual delivery of treatment via intra-cisterna magna (ICM) and intravenous (IV) routes, along with the ancillary administration of immunosuppressant drugs. Treatment for 10 cohorts of 10 mice involved concurrent infusions through both ICM and IV routes. There were three possible infusates for the ICM route (vehicle, low or high vector dose) and six possible infusates (vehicle, 5 different vector doses) for the IV route. The study design allows an efficacy comparison of the total dose when administered either through one route of delivery or split between the two. The researcher who conducted the procedure and subsequent testing is blinded to which dosage each animal received. Bimonthly behavioural testing and blood collections at specific time points were done until mice reach their humane endpoint as determined by specific UACC criteria. At termination, blood, gross organs, brain, and spinal cord were collected for analysis of GM2 ganglioside accumulation, vector copy number, Hex enzyme activity, cellular and humoral immune response, and histology. To date the results from the long-term study show increased survival in all treatment groups compared to the vehicle-only control group ($p < 0.001$ in all cases). The treatment cohort with the longest survival showed a >6.5-fold increase in median survival compared to the control group. Additionally, some treated cohorts showed a >3-fold decrease in accumulated gangliosides compared to controls in the mid-section of the brain ($p < 0.01$). In sera collected at humane endpoints there was a >51-fold increase in Hex enzyme activity in the longest living treatment group compared to heterozygous controls ($p < 0.05$). Ganglioside accumulation in the cerebellum, Hex activity in the brain, vector biodistribution, and behavioural data will also be shown.



* Cohort T is the untreated (vehicle only) cohort, unblinded by virtue of their early death typical of SD mice.

891 Development of a Competitive Ligand-Binding Assay to Detect Neutralizing Antibodies Against Chimeric Antigen Receptor of Regulatory T Cells

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Regulatory T cells (Tregs) have emerged as a potential treatment modality for various types of diseases. Tregs expressing chimeric antigen receptor (CAR) is being evaluated clinically for the treatment of human leukocyte antigen (HLA)- mismatched organ transplant rejection. Host immune response against the CAR protein represents a risk to clinical translation of CAR-T cell therapies due to its potential impact on safety and efficacy. The anti-CAR humoral immune response can generate antibodies that cause rapid clearance of CAR-T cells and may also neutralize CAR-Treg function. Therefore, development of appropriate assays to monitor and characterize anti-CAR antibodies is recommended by regulatory agencies and can ensure proper clinical development of the CAR-Treg cell products. Anti-CAR total antibodies (TAbs) have been detected using ligand-binding and cell-based assay formats. However, it is challenging to develop anti-CAR specific neutralizing antibody (NAb) assays, especially for the humanized CAR constructs due to the lack of commercially available assay reagents. This study will present the development of a competitive ligand-binding assay to detect NAbs against an engineered CAR, which is comprised of a humanized single-chain variable fragment (scFv). This scFv is designed to enable CAR Tregs to recognize and bind to the HLA-A2 antigen on donor kidney cells in HLA-A2 mismatched kidney transplantation patients. As a positive control, an antibody against the scFv was generated by rabbit single B-cell cloning technology and was able to specifically block the binding of the scFv to HLA-A2 *in vitro*. Using this anti-CAR NAb positive control, we evaluated both ELISA and MSD platforms and selected the MSD method based on better assay performance. The optimized MSD assay yielded a sensitivity of 141 ng/mL. Regarding drug tolerance, the assay detects 181 ng/mL of the positive control in the presence of 400 ng/mL of free scFv. In addition, we will discuss the challenges and strategies to implement cell-based anti-CAR NAb assays and improving NAb assay drug tolerance for tissue-targeting CAR-Treg cell therapy.

892 Engineering Non-Human Primate B Cells for Expression of Broadly-Neutralizing Anti-HIV/SIV Antibodies

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No vaccination strategy for HIV has been successful, in part because the antibodies that are induced are unable to neutralize the wide variety of circulating HIV subtypes, and resistance can easily be acquired by HIV mutation. In contrast, a small number of broadly-neutralizing antibodies (bnAbs) have been identified that provide good viral control, especially when used in combination. However, the use of recombinant bnAbs as an anti-HIV therapeutic would be costly and require life-long injections. As an alternative, we have previously described a genome editing approach at the IgG1 locus that results in reprograming of human B cells to express customized heavy-chain antibodies (HCAbs). HCAbs mimic a natural antibody type expressed by camelids and can be engineered with a variety of antigen-recognizing domains, including VHHs, scFvs, and other protein domains. Importantly, HCAb edited human B cells retain normal B cell functions such as clonal expansion and somatic hypermutation in response to antigen. Moreover, insertion of anti-HIV bnAb domains results in the secretion of HCAbs that neutralize a broad panel of HIV isolates. We hypothesize that such cells will be capable of long-lived engraftment and HIV control, including by providing B cell memory that could be boosted over time. To test the potential of HCAb-engineered B cells as a prevention or therapy against HIV, the next step is to adapt our editing approach for the Ig locus in rhesus macaques. These animals are useful for studies of HIV, and can be infected by either natural SIV isolates or hybrid SHIVs that substitute an HIV Envelope protein into an SIV backbone. In addition to our panel of broadly neutralizing anti-HIV HCAbs, we have now also derived novel antigen-binding domains that recognize the SIV Env. These include scFvs derived from recently identified anti-SIV bnAbs and a rationally designed variant of the receptor mimic, eCD4-Ig, that is compatible with this genome editing protocol. In parallel, we have optimized protocols necessary to isolate, activate and engineer the macaque B cells, to produce a cell product suitable for autologous transplantation. Together, these reagents and protocols are a useful toolkit for B cell engineering in rhesus macaques, and will allow us to test the ability of HCAb-edited B cells to protect against SIV/SHIV infection.

893 Preclinical Workup for an Approved Phase I/II Clinical Trial for Mucopolysaccharidosis II (MPSII): Validation of a GMP Stem Cell Gene Therapy Manufacturing Process

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Hematopoietic stem cell gene therapy (HSCGT) is a promising therapeutic strategy for the treatment of metabolic disorders where the central nervous system (CNS) is severely impaired. An HSCGT treatment developed for early-onset metachromatic leukodystrophy (MLD), a disease caused by mutations in the arylsulfatase-A gene, demonstrated successful outcomes in clinical trials and was recently approved for use in the UK and Europe. The HSCGT approach utilises a lentiviral vector to genetically modify patients' own stem cells *ex vivo,* introducing correct copies of a defective gene. Following transplantation back into a fully conditioned patient, these genetically modified autologous hematopoietic stem cells (HSCs) can repopulate the blood system, with a subset differentiating into myeloid cells that can traffic to the brain. Crucially, functional protein can be delivered to affected cells in both somatic organs and the central nervous system with the potential to alleviate peripheral and neurological disease symptoms and halt disease progression. We have developed a HSCGT approach for the treatment of Mucopolysaccharidosis type-II (MPSII, Hunter Syndrome), a debilitating paediatric lysosomal disorder caused by mutations in the iduronate-2-sulphatase (*IDS*) gene, leading to accumulation of heparan and dermatan sulphate which causes severe neurodegeneration, skeletal abnormalities and cardiorespiratory disease. For this application we developed a lentiviral vector that expresses human IDS fused to the blood-brain barrier crossing peptide ApoEII (LV IDS.ApoEII) to facilitate delivery of IDS to the CNS by two independent mechanisms; cell-mediated transcytosis and receptor-mediated transcytosis. In proof-of-concept (PoC) studies, HSCGT using LV IDS.ApoEII was able to normalise brain pathology and behaviour of MPSII mice, providing significantly enhanced correction compared to mice receiving HSCGT with a lentiviral vector expressing IDS only or treatment with a conventional allogeneic bone marrow transplant. Following these successful PoC studies we have been developing MPSII HSCGT for clinical application. Here we have validated a clinical stem cell transduction protocol for MPSII on peripheral blood derived human CD34+ cells, with inclusion of transduction enhancers protamine sulphate and LentiBOOST™ in order to reduce vector dose, whilst comparing and contrasting different culture conditions. Two manufacturing validation runs utilising IL-3 in the transduction media with a vector dose of MOI25 resulted in vector copy numbers of 5.09 and 6.34, and 97.1% and 98.1% cell product viability respectively. In pilot manufacturing runs with IL-3 absent from the transduction media, transduction efficiency was reduced 3-4 fold. Inclusion of IL-3 however, can initiate HSC differentiation which could potentially affect downstream cell engraftment. In two GMP pilot manufacturing runs using a vector dose of MOI100 and no IL-3, VCNs of 4.73 and 2.25 were achieved with 91.79% and 91.63% cell

product viability and high levels of human IDS activity of 1155.2 and 1511.9 μM 4-MU/4hr/μg respectively in CFU cultures seeded from the transduced cell product. Following these preclinical validations, we recently acquired MHRA regulatory approval for a phase I/II HSCGT clinical trial in MPSII patients due to start recruitment in 2023.

894 Development of a Trans-Splicing AAV Vector for the Treatment of Neurofibromatosis Type-I

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Neurofibromatosis Type 1 (NF1) is a neurologic disease is a neurologic disease with a complex pathological burden leading to substantial cognitive and neurologic dysfunction with neuro-oncologic manifestations. As a single gene loss-of-function disorder where more than 50% manifestations are caused by sporadic mutations, NF1 is a very attractive candidate for a gene replacement type of therapy. The large size of the NF1 cDNA (~8.5 kb) is a considerable challenge for development of AAV-based gene therapies for this disease. Our laboratory has developed two AAV vector systems, a dual system with the cDNA split across two AAV genomes and a mini-NF1 gene capable of regulating Ras activity. We are developing an RNA editing using spliceosome mediated RNA trans-splicing (SMaRT) approach for NF1. SmaRT utilizes hybridization of a vector encoded mutation free mRNA, also known as a pre-trans-splicing molecule (PTM), to the endogenous mutated pre-mRNA, thereby providing the splicing machinery with alternative splice signals. The parameters that make a successful pre-trans-splicing molecule (PTM) are largely unknown, and because of that we are using a library approach to screen for the binding domains that have the greatest trans-splicing efficiency. To achieve this, we tiled 150 bp sequences by 1 nt through the adjacent 3'-intron and immediate 3'-exon corresponding to the location we wish to splice (NF1 Exon 32)(Fig. 1).

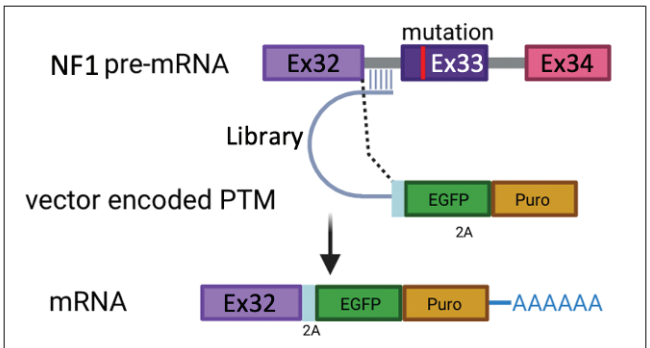

Figure 1. Selection of PTMs for NF1

This resulted in a library composed of 1,251 PTMs that could potentially enable the binding and successful trans-splicing of our vector encoded molecules to the endogenous NF1 mRNA. We designed a lentiviral vector system encoding the PTM library followed by a splice acceptor signal, and the 3' NF1 exon fused to a EGFP-Puromycin cassette via a 2A peptide to select for positive events. NGS analysis of the plasmid library indicated a 121x coverage for each PTM. Lentivirus stocks were produced and used to infect HEK293T cells, and puromycin selection started at 48hrs. Infected cells were expanded under selection for two weeks when gDNA and RNA were collected, and single clones selected for expansion and targeted analysis. Single clone analysis revealed two different inserts with clear evidence of trans-splicing from the endogenous NF1 sequence to our vector encoded sequence. NGS analysis of pooled selected cells indicated a mapping of inserts to coverage "hot-spots" within the 3'-intron indicating regions of variable strength in trans-splicing. The two inserts from single clones individually mapped to a region with high coverage and a region with low coverage. We are currently exploring the efficiency of trans-splicing with those clones as well as individual selections. Once optimal inserts are chosen, they will be encoded into vectors containing the remainder of 3' NF1 and tested in their ability to restore NF1 expression and function in NF1 patient cell lines. Eventually this work will pave the way for a semi-customized gene therapy for NF1 where patients can be given one of three vectors to only replace the portion of their NF1 which is mutated. This simplicity of design would allow for broad application of the vectors to many patients and maintaining the endogenous control of NF1 will abrogate many issues that can arise with gene dosage in similar gene replacement therapies.

895 Viral Clearance Strategies for Gene Therapy Products

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The adeno-associated virus (AAV) is a common delivery vehicle for gene therapy products. For all biotechnology products derived from cell lines of human or animal origin, there is a risk of viral contamination from the source material or from adventitious agents during the production process. Adventitious agents, including new and emerging viruses, can be unintentionally introduced and enter a production process from different routes. It is important to assess the potential impact of adventitious agents or viruses on the safety profile of AAV gene therapies and other advanced therapy medicinal products. To ensure product safety, testing programs should take an integrated and comprehensive approach to reduce the risk of contamination across the entire manufacturing process. Developing a robust viral clearance program requires a careful assessment of risk and a thorough understanding of the manufacturing process used to develop the gene therapy product. Keeping in mind the unique properties of AAV gene therapies and other advanced therapy medicinal products, dedicated unit operations for viral clearance will typically involve virus removal or virus inactivation. Virus removal can be accomplished by chromatography or nanofiltration/virus filtration, while virus inactivation can occur via a variety of treatment methods. For gene therapy products, various chemical treatments may be utilized including the use of low pH, solvents, detergents, or a combination of solvent and detergent. Process evaluation studies were designed to test the inactivation of virus from spiked test articles

during execution of process steps. For each scaled-down process step, virus was intentionally spiked into the starting material or load, the process was performed, and the amount of virus present in samples collected at the start and end of the process was quantified. The capacity of each step for inactivation of virus was calculated and reported as the log reduction value.

896 Esophageal Cancer-Specific Gene Therapy with Liposome-Processed Oncolytic Adenovirus Introduced with SCCA1 Gene Promoter

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The incidence of esophageal cancer is increasing, and the survival rate of advanced cancer is around 40-50% with no improvement in prognosis. The esophagus is an important functional organ related to daily eating and swallowing. Because of the deterioration of swallowing function due to surgery and radiation, hoarseness due to recurrent nerve paralysis, and the presence of important organs such as the heart and lungs in the surrounding organs, it is necessary to preserve the function of these organs during treatment. Gene therapy is a non-invasive treatment that can be cured by injection alone, injected endoscopically and administered multiple times. This makes it a good indication for early-stage, locally advanced and recurrent cancers. SCCA1 is expressed specifically in squamous cell carcinoma and is a good tumor marker for esophageal cancer with squamous cell carcinoma in more than 90% of cases. We cloned the SCCA1 gene promoter and was introduced into an oncolytic adenovirus AdE3-SCCA1. AdE3-SCCA1 has an antitumor effect specifically on squamous cell carcinoma, but infection is suppressed because of anti-adenovirus antibody. Therefore, oncolytic adenovirus was coated by liposome processing to overcome immunogenicity and its antitumor effect was investigated. The antitumor effect of liposome-coated AdE3-SCCA1 on murine squamous cell carcinoma SCC7 cells of C3H/HeN syngeneic mouse model was investigated. In the presence of anti-adenovirus antibodies, AdE3-SCCA1 is completely inhibited from infection, but liposome processing made it 80% infectious. Liposome-processed AdE3-SCCA1 showed no antitumor effect on SCC7 tumors in non-preimmunized mouse, but on adenovirus-preimmunized mouse, complete tumor regression was achieved against 70% of subcutaneous tumors by anti-adenoviral CTL induction. Liposome-processed oncolytic adenovirus AdE3-SCCA1 was suggested to be an effective therapy against esophageal cancer.

897 Evaluation of Affinity and Ion-Exchange Chromatography-Based Platforms for Consecutive Purification of AAV9 Vectors

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Adeno-associated virus (AAV) vectors are leading delivery systems for pre-clinical and clinical gene therapy. However, difficulties in manufacturing including processing (host cells proteins, DNA, residual plasmid, helper virus genes, endonucleases) and product-related impurities (empty and partially full capsids, size variants, ununiform post-translational modifications, degradation) still need to be resolved. Particularly, the inefficient separation of empty capsids constitutes the vital part of an AAV preparation that pose a risk on clinical safety and effectiveness of AAV-based gene therapy. Hence, the development of selective, efficient, reproducible, scalable and cost-effective purification methods for the separation of empty capsids is vital for AAV manufacturers. Density gradient ultracentrifugation is the common and long-used method, needs to accomplish some drawbacks including time-consuming process nature, limited sample loading capacity, scalability, and the requirement for additional clarification, dialysis, ultrafiltration steps to achieve a resulting preparation with expected purity. Therefore, the use of chromatography-based methods might facilitate clinical-scale AAV production. In the current study we evaluated several affinity followed by ion-exchange chromatography-based separation platform to obtain high AAV titer and superior full to empty capsid ratio. AAV9 harboring firefly luciferase gene flanked by ITRs was produced in adherent HEK293 cells by triple transfection method. Harvested cells were disrupted by freeze-thaw cycle and cellular debris was removed by brief centrifugation. Supernatant consisting of cellular components and viral capsids was loaded on AAV9-specific AVIPure® affinity purification column. Subsequently, the eluted AAV9 capsids were divided equally and further purified by on AKTA™ chromatography system with HiTrap™ Q HP, HiTrap™ Capto™ Q and CIMmultus™ QA ion- exchange columns. BTP/$MgSO_4$ (pH 9) buffer-was used for the purification with HiTrap Q and CIMmultus™ QA columns, and BTP/$MgCl_2$/$C_2H_3NaO_2$ (pH 9) buffer was used for HiTrap™ Capto™ Q column. Step gradient elution was applied to collect enriched AAV fractions. Viral genome titer of recovered AAV vectors were quantified by qPCR, and efficiency of encapsulation was evaluated by TEM imaging. We achieved over 95% of full/empty capsid ratio and about 30 % recovery with HiTrap™ Q HP column. In contrast CIMmultus™ QA column improved both full/empty capsid ratio over than 98% and the recovery up to approximately 70% as and calculated full/empty capsid ratio on HiTrap™ Capto™ Q column was over 90% and recovery approximately 80%. Additionally, the infectivity of fractions recovered from different ion exchange columns were also evaluated by in vitro luciferase assay on HEK293 cells transduced with MOI 20,000 vg/cell. AAV-mediated luciferase activity normalized to viral titer was similar for all purified fractions. Based on the results of we concluded that electrostatic interaction between different column material and viral capsid does not have a detrimental effect AAV vectors ability to interact cellular receptors. Our preliminary data obtained from purification on three different ion exchange columns following an affinity capture indicate that tandem chromatography might be effective method to obtain high titer and high purity AAV vectors.

898 Hepatocyte Evolution During Post Natal Growth Affects Lentiviral Gene Transfer and Its Biodistribution in the Liver Lobule

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The liver is an attractive target organ for *in vivo* gene therapy since it offers the possibility to treat metabolic and coagulation disorders. Promising pre-clinical and clinical results have been achieved in adult patients treated with adeno-associated viral (AAV) vectors, however, their use in pediatric patients is challenged by their non-integrating nature and consequent dilution of the episomal genome upon cell proliferation during liver growth. We developed lentiviral vectors (LV), which integrate into the cell genome, targeting transgene expression into hepatocytes, and achieved stable coagulation factor IX (FIX) expression in adult mice, dogs, and non-human primates. Here, we investigated efficiency of LV-based liver gene transfer in young mice, towards potential application to pediatric patients. We administered LV expressing FIX as a secreted reporter transgene to mice at different ages and observed higher FIX levels in young-treated mice (newborn or 2-week-old) compared to adult-treated mice (8-weeks-old). We isolated hepatocytes 3 days after treatment and measured a higher vector copy number (VCN) in young-treated mice compared to adults, paralleled by a lower VCN in non-parenchymal cells, particularly Kupffer cells, suggesting that lower phagocytic activity plays a role in determining a higher transgene output in young mice. Indeed, when we administered LV expressing a fluorescent reporter transgene to mice of different ages, we observed a 4-fold higher LV-positive area in the liver of young-treated mice compared to adults. Moreover, we noted non-homogeneous distribution of LV-marked hepatocytes in the liver lobule, with preferential transduction of the peri-central area in young-treated mice and peri-portal area in adult-treated mice. We then performed spatial transcriptomics of livers of young and adult mice to analyze changes in transcriptome profile that could impact hepatocyte permissiveness to LV transduction. In young mice, hepatocytes showed a peri-portal transcriptional profile, regardless of their localization in the lobule, lacking a fully established metabolic zonation. We thus hypothesized that peri-central may be less permissive to LV transduction. The proteasome pathway resulted to be more expressed in adult compared to young livers and peri-central compared to peri-portal hepatocytes. Thus, we administered the proteasome inhibitor bortezomib to adult mice before LV and achieved higher and more homogeneous liver transduction compared to LV-only treated mice, indicating that proteasome plays an important role in determining the efficiency and distribution of hepatocyte transduction by LV. Finally, we evaluated proliferation of LV-transduced and untransduced hepatocytes, by administration of EdU at different ages during growth. EdU is incorporated during DNA synthesis and allows marking of proliferating cells. We observed that 20% of hepatocytes were EdU-positive in young mice, while proliferation was almost absent in adults. We observed the same percentages also for transduced hepatocytes, indicating that only a fraction of hepatocytes proliferate during post-natal growth and LV transduction does not affect hepatocyte proliferation rate. Overall, our results indicate that LV-based liver gene therapy is more efficient in young mice, and that age of treatment impacts on LV transduction efficiency, its spatial distribution in the liver lobule and transgene output. These studies will inform further development of liver-directed LV gene therapy towards application to pediatric patients.

899 Molecular Shielding of the Pan-Hematopoietic Marker CD45 May Enable a Universal Approach for Replacement of the Hematopoietic System

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CD45-targeted surrogate antibody-drug conjugates (ADCs) enable chemotherapy-free hematopoietic stem cell transplantation (HSCT) in mice. However, the pan-hematopoietic marker CD45 is critical for the function of immune cells as illustrated by mutations underlying severe combined immunodeficiency (SCID). Therefore, CD45-targeted cell depleting therapies are currently limited to a single dose. Yet, treatment of malignancies or autoimmune diseases may require longterm therapy with CAR T cells or repeated dosing with ADCs. We recently demonstrated that single amino acid substitutions engineered into CD123 or CD117 expressed by hematopoietic stem and progenitor cells (HSPCs) protected the cells from cytotoxicity by matching immunotherapies but preserved CD123 and CD117 function, respectively. Here, we describe the identification of a CD45 shielding variant paired with the development of a new and potent CD45-targeting ADC (CIM053-ADC). To overcome limitations of homology directed repair (HDR) we aimed to install the molecular shield using base editors (BE). We computationally selected extracellular CD45 regions and screened sgRNA/base editor combinations that may generate cell shielding amino acid substitutions. We identified multiple base editable substitutions that reduced or abolished the binding to different anti-CD45 antibodies while preserving binding to control antibodies. Base edited human primary T cells were shielded from surrogate antibody-drug conjugate (ADC) killing in vitro resulting in enrichment of base edited shielded cells while wild-type cells were progressively depleted with increasing ADC concentrations. Thus, combining a shielding variant with a paired ADC resulted in a user-controlled ratio of unedited:edited cells in vitro. However, biophysical characterization of the top CD45 variant proteins revealed limitations. One CD45 candidate variant that displayed residual binding resulted in incomplete shielding while others demonstrated altered protein properties. In one case this was partially attributable to a bystander edit introduced by the BE. An expanded and optimized screen identified a lead variant which completely shielded >50% HSPCs from the binding by CIM053 while maintaining intact protein properties. Co-culture with MOLM-14 or Jurkat tumor cells (i.e. representing different cell lineages) in the presence of CIM053-ADC selectively depleted

MOLM-14/Jurkat and unedited HSPCs but left shielded HSPCs intact in vitro. Furthermore, CIM053-ADC potently depleted HSPCs in mice reconstituted with human CD34+ HSPCs while being well tolerated. Thus, we identified a base editable CD45 variant that shields from a monoclonal antibody with favorable characteristics for clinical development. The combination is ready for testing whether it enables tumor-selective posttransplant immunotherapy in mice xenografted with human HSPCs.

900 Evaluation a Novel Fluorescence-Based Approach to Sensitive Bacterial Endotoxin Testing

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Endotoxin contamination is a common problem with recombinant proteins and nucleic acids used in cell and gene therapy. These heat-stable lipopolysaccharides (LPS) are associated with the outer membranes of certain gram-negative bacteria and often introduce themselves into the experimental workflow during the lysis of plasmid DNA preparations. In cell-based experiments, contamination with endotoxin can trigger toxic shock, activation of nonspecific immune responses, or premature cell death. These effects can significantly compromise cell-based studies and skew experimental results. Current approaches to endotoxin testing primarily utilize amebocyte lysate in which an enzymatic cascade is activated in the presence of endotoxin to produce a colorimetric or turbidimetric readout. These assays are a mainstay of endotoxin detection, but often require cumbersome workflows, cost-prohibitive instrumentation, and lengthy manual data analysis. Here we describe and evaluate a novel approach to endotoxin detection which utilizes a fluorogenic reporter to improve sensitivity - offering a 0.01 - 10 EU/mL detection range with a workflow that is more streamlined than existing approaches. Furthermore, we demonstrate the impact of endotoxin contamination in a model cell study where known amounts of endotoxin with a GFP encoding plasmid are titrated into primary cells. The results highlight the effects of increasing endotoxin levels on transfection efficiency, cell line morphology, and overall cell health.

902 A Robust and Scalable Research Grade Platform Process for AAV6 Production and Purification

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The field of gene therapy using adeno associated virus (AAV) is making significant strides as a novel therapeutic with the potential to treat and cure monogenetic disorders. AAV can be engineered to deliver long-term transgene expression cassettes to target cells, with low risk of integration and pathogenicity. With hundreds of clinical trials underway, there is a clear need for robust, scalable, and cost-effective platforms to generate research-grade material for proof-of-concept work that is more closely representative of process development platform material. We describe here a platform process for AAV6 production using a transient triple transfection, helper-free system, with serum-free suspension cells, followed by affinity resin purification using a batch method. Using an AAV transfer vector containing a 4.7kb insert comprising a representative gene knock-in cassette, we were able to achieve titers around 1E11 VG/mL for crude production and a 60% yield after affinity batch purification in our research grade platform. Both the upstream AAV6 production and downstream affinity resin batch purification align very closely with our larger scale process development platform. This allows for faster, quicker transition from research to development projects. AAV6 vector preps made with both research grade and process development show comparable transduction efficiency on HeLa cells with >90% and >50% in B cells 48 hours post transduction, at MOIs as low as 5e4 (VG/cell). No overt signs of toxicity were observed at MOIs as high as 5e5. This process will allow for an easy transition from research to process development teams for GMP manufacturing purposes.

903 An Engineered Beta-galactosidase with Improved Stability and Cross-Correction for the Potential Treatment of GM1 Gangliosidosis

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GM1 gangliosidosis is an autosomal recessive deficiency of β-galactosidase (β-gal) characterized biochemically by the accumulation of GM1 ganglioside and its asialo derivative GA1 in the central nervous system. Accumulation of gangliosides and glycosaminoglycans (GAGs) leads to the progressive loss of neurons in the brain and spinal cord with concomitant peripheral tissue pathologies. No therapy is currently available for this lysosomal storage disease, and lethal by age 3 in the most severe early-onset cases. While gene therapy delivering *GLB1* to the central nervous system offers potential to slow or halt disease progression and potentially correct harmful phenotypes in patients, intrinsic limitations of the wild-type β-gal enzyme may limit the effectiveness of this approach. For example, while cross-correction of un-transduced neighboring cells and deep brain structures is required to broadly rescue storage deficits in the CNS, β-gal rapidly and irreversibly degrades at neutral pH and in body fluids. To address these limitations, we applied the CodeEvolver® protein engineering platform to screen >10,000 β-gal variants over five rounds of iterative directed evolution. The most promising β-gal variants identified are ≥20-fold more stable than wild-type β-gal in serum and artificial cerebrosidal fluid (CSF). We demonstrate that the engineered β-gal variants are taken up by and exhibit activity in GM1 gangliosidosis patient fibroblasts and human iPSC-derived GM1 gangliosidosis forebrain neurons at ≥10-fold over wild-type β-gal, potentially enabling better cross-correction capacity. This increase in stability and cellular uptake does not impact neuraminidase activity, suggesting a lack of enzymatic over-correction. Finally, we show data on the delivery of the engineered β-gal transgenes in AAV9 to GM1 ex vivo cellular disease

models and to GM1 gangliosidosis mice. Collectively, these data show the promise of an optimized β-gal transgene as a potential gene therapy for treating patients suffering from this devastating disease. **Keywords:** GM1 gangliosidosis, protein engineering, directed evolution, lysosomal storage disease, gene therapy

904 Development of Capsid + Genome-Modified Optimized (Opt^{Y}) AAVrh74 Vectors with Improved Transgene Expression in Mouse Skeletal Muscles Following Systemic Administration *In Vivo*

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We have previously reported that capsid-modified next genaeration triple-mutant (TM) AAVrh74 vectors, in which surface-exposed tyrosine (Y733 and Y447) and threonine (T494) were mutagenized, transduced primary human skeletal muscle cells more efficiently than the wild-type (WT) AAVrh74 vectors *in vitro* (*Mol. Ther.*, 29: 159-160, 2021). Similarly, we have also reported that genome-modified generation Y (GenY) AAVrh74 vectors, in which the distal 10-nts in the D-sequence were replaced with the 15-nts consensus glucocorticoid response element (GRE) site, transduced primary human skeletal muscle cells more efficiently than the WT AAVrh74 vectors *in vitro* (*Mol. Ther.*, 30: 237-238, 2022). In the current studies, we evaluated whether the combination of the two, resulting in optimized (Opt^{Y}) AAVrh74 vectors, would lead to an additive effect. GenY genomes were packaged into TM capsids to generate Opt^{Y} AAVrh74 vectors, and the transduction efficiency of these vectors was evaluated in mouse gastrocnemius (GA) and tibialis anterior (TA) muscles following systemic delivery in C57BL/6 mice (N=6 each). Transgene expression was evaluated 8 weeks post-injections. These results, shown in **Figure 1A**, indicate that compared with the TM vectors, transgene expression from the Opt^{Y} vectors, both in TA and GA muscles, was higher (**Figure 1B**). We also evaluated the extent of the transgene expression in mouse liver, heart, diaphragm, and GA and TA muscles at the RNA level. The Delta-Delta analysis, relative to the PBS control (**Figure 1C**) and to the TM AAVrh74 vector control (**Figure 1D**), indicate that the extent of the transgene expression from the Opt^{Y} AAVrh74 vectors was significantly higher in each of the muscle tissues, but not in the liver. Taken together, these studies suggest that the combined use of the capsid-modified NextGen + genome-modified GenY (Opt^{Y}) AAVrh74 vectors may further reduce the need for high vector doses currently in use, which has significant implications in the potential use of Opt^{Y} AAVrh74 vectors in the safe and effective gene therapy of muscular dystrophies in humans. This research was supported by a sponsored research grant from Sarepta Therapeutics.

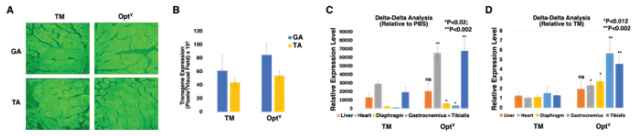

Figure 1: Transduction efficiency of TM and Opt^{Y} AAVrh74 vectors in primary murine muscles *in vivo*. A. Each vector was injected intravenously at 1x10^{12} vgs/mouse and transgene expression in tibialis anterior (TA) and gastrocnemius (GA) skeletal muscle sections was visualized under a fluorescence microscope 8-weeks post-injections. **B.** Data were quantitated using the ImageJ software. Total RNA samples isolated from various organs were subjected to RT-PCR-qPCR and analyzed by ΔCt = (hrGFP Ct – β-actin Ct) respective average WT-AAVrh74 ΔCt and reported as relative expression level or $2^{-\Delta\Delta CT}$ relative to PBS (**C**) and to TM-AAVrh74 vectors (**D**), respectively.

905 Novel Adenoviral Vectors for Gene Therapy Approaches to Treat Cerebellar Disorders

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Genetic variations are major risk factors for a multitude of cerebellar disorders, which can manifest in tissue-wide or cell type-specific dysfunction and degeneration. Cerebellar disorders are largely due to mutations in genes that particularly affect Purkinje cells (PCs), which are the neurons that mediate the sole output of all motor information processing in the cerebellar cortex. Due to the monogenic nature of CACNA1A disorders and Ataxia Telangiectasia (A-T), they are ideal candidates of cerebellar disorders to treat with viral vector gene therapy approaches. However, the cDNA size of the underlying *CACNA1A* and *ATM* genes exceed the packaging limits of AAV and Lentiviral vectors (LV). Therefore, non-toxic viral vectors with large carrying capacities capable of long-term stable transgene expression in PCs are needed. Helper-dependent Adenoviral vectors (HdAd) are safe, high-capacity vectors that overcome the packaging limitations of AAV and LV. Currently, the most common Adenoviral vectors are of serotype 5 (Ad5) and infect cells via Coxsackie-Adenovirus Receptor (CAR). However, PCs are refractory to CAR-dependent Ad5 infection. To overcome the limitation of Ad5, we developed Ad vector variants that use an alternative human receptor. We characterized these vectors in the cerebellum of a humanized mouse model and identified variants with tropism for PCs and other cerebellar cell types. The development of these vectors greatly expands the utility of the HdAd platform for treating CACNA1A disorders, A-T, and other disorders that will require expression of genes or transgene cassettes exceeding the capacities of AAV or LV. Finally, we propose that our vectors will have immediate translational applicability to humans for cerebellar and other Central Nervous System disorders.

906 Evaluation of a Scalable Harvest Protocol Using a Panel of AAV Serotypes with Attention to Recombinant AAV Vector Integrity and Functionality

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To date recombinant adeno-associated viral vectors (rAAV) have delivered functional gene copies in over 200 clinical trials and are the drug substance in five FDA or EMA approved products. There is a continued effort to design more efficacious rAAV vectors, aiming to e.g., reduce vector doses. However, several indications require systemic administration at currently still high rAAV vector doses, making rAAV manufacturing and upscaling paramount to continue rAAV's success story. Here, we aim to evaluate a more scalable harvest protocol, compared with our current standard protocol and its impact on vector integrity as well as functionality on various capsids. The rAAV vectors rAAV1-9 and rAAV-PhP.eB were produced at 30 ml scale in the AAV-MAX suspension cell system. For triple transfection, the same rAAV transfer plasmid (pAAV-CMV-EGFP) and adenoviral helper plasmids were used, as well as analogously built Rep/Cap plasmids. Subsequently, each production was either subjected to the scalable or the standard harvest protocol (n=3). The scalable protocol uses the AAV-MAX lysis buffer (ThermoFisher) which can be added directly to the cell suspension for lysis. In the standard protocol, the cell suspension is spun down and the cell pellet is lysed, while the rAAV from the supernatant is precipitated. The resulting crude lysates were analysed for vector genome (VG) titers using qPCR targeting the ITRs, with free-ITR standard curve and ATCC's AAV2 reference standard material as control. The scalable harvest protocol resulted in titers in the 6.6E11-1.1E12 VG/ml range and the standard protocol in the 4.6E11-1.9E12 VG/ml range. Comparing across vectors, the scalable harvest protocol led to lower crude lysate titers compared with the standard protocol (mean decrease 22 %, SD 9.2 %), except for rAAV4 where the scalable protocol led to increased titers. For further quality control, crude lysate triplicates from the scalable harvest protocol were purified using the POROS GoPure AAVX/9 affinity chromatography column. To determine the recovery after affinity chromatography (n=3), the elution was titrated as described above. Vectors with AAV serotypes 2, 3, 5 and 8 achieved high recovery levels (mean recovery>80 %), serotypes AAV1, AAV6 and PhP.eB achieved medium recovery levels (60<mean recovery<80 %) and AAV4's recovery rate was low with 42 %. Next, vectors were analysed for empty/full capsid ratio using the Stunner Instrument (Unchained Labs, n=3). The serotypes AAV2, 5, 8, and PhP.eB showed lower levels of full capsids (11-18 %), whereas the serotypes 1, 3, 4, 6 and 9 had a high proportion of full capsids (30-55 %) which was an evaluation of upstream process (USP) packaging efficiency. The capsid aggregation was measured in PBS buffer using dynamic light scattering (Stunner instrument, Unchained Labs, n=3). Aggregation was lower than 2 % for all serotypes, except AAV6 and AAV2. The observed empty/full ratios as well as the aggregation levels matched previous experience. We concluded that the scalable harvest protocol resulted in lower USP vector yields but achieved comparable vector quality. Taking the ease of upscaling into account, the USP yields in the scalable protocol are deemed an acceptable first result. Further optimisations are underway to increase USP yields. To test the impact on vector functionality, the next step is comparing a panel of vectors either harvested with the standard or scalable harvest protocol in an *in vitro* assay. The learnings from these process optimizations have the ultimate aim to inform rAAV vector production for large scale clinical applications in a cost-effective fashion.

907 Targeted Genome Integration in a Novel Muscle-Specific Safe-Harbor Site Enabling High Transgene Expression

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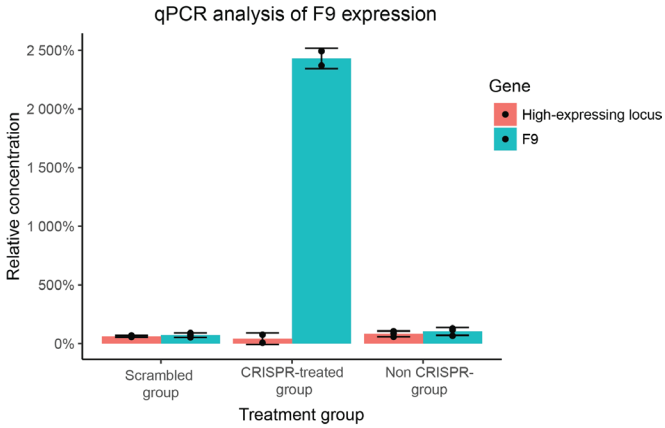
Targeted integration has emerged as a gene editing technique to treat a broad range of genetic diseases and expand therapeutic options of synthetic biology. Despite substantial progress made in targeted gene delivery methods, identifying safe loci to insert replacement genes for optimal safety and efficiency remains a challenge. Gene editing approaches often target the disease locus itself; however, the proportion of successfully integrated genes may not produce the protein levels needed to alleviate the phenotype, and semi-random viral-mediated integration could lead to mutagenesis. An exciting alternative would be to identify a safe-harbor locus with high transcriptional activity. This research aims to investigate new potential sites that can be used for safe and stable integration. Here, we proposed and validated a high-expressing locus in skeletal muscle as a target site to insert a promoterless reporter gene or human factor 9. We co-delivered CRISPR-Cas9 and a homology-independent targeted integration (HITI) donor insert plasmid with a panel of candidate splice sites to hijack the endogenous promoter of muscle-specific loci in myoblasts. As a proof of concept, in our early validation with lipid nanoparticle delivery, we achieved precise in-frame integration of green fluorescent protein (GFP) and human factor 9 in mouse DNA and mRNA by genotyping the targeted site in mouse myoblast cells using primers spanning the junctions between the 5' end of the integration site and the 3' end of the insert. We also demonstrated the precision of integration of GFP at 60% at the DNA level and up to 96% at the mRNA level using Illumina next-generation sequencing. This result indicates that integration at intronic regions reduces unintended modifications during transcription and translation. To further investigate the precision of integration, we performed a high throughput short- and long-read sequencing using a unique molecular identifier-tagged library to eliminate the bias of PCR-enriched quantification efficiency. At the RNA level, the integration of human factor 9 in the muscle-specific locus showed an increase of relative expression up to 2,500-fold compared to scrambled gRNA and non-CRISPR treated group in mouse myotubes by qRT-PCR analysis (Figure 1). Additionally, a series of our studies are aimed at characterizing gene integration

safety and therapeutic efficacy in mouse models. Furthermore, global transcriptome changes are assessed by RNA sequencing. By integrating a therapeutic gene under an endogenous muscle-specific promoter, this strategy offers a safer route of administration and high expression of therapeutically relevant proteins in circulation. This approach also provides a promising framework for advancing mutation-independent gene therapy and a platform for *in vivo* synthetic biology.



908 Delivering Genes to the Brain Endothelium to Treat Lysosomal Storage Disorder-Derived Neuropathology

Nuria Roxana Romero Navarrete, Jason Wu, Thomas Beddow, Benjamin E. Deverman

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Lysosomal storage disorders (LSDs) are genetic diseases characterized by the absence of functional lysosomal enzymes or transporters. The ensuing accumulation of lipids, glycosaminoglycans (GAGs) or glycoproteins results in damage to cells and tissues such as the central nervous system (CNS), which can lead to cognitive impairment and premature death. Enzyme-replacement therapy is the standard of care for many LSDs but the inability of recombinant enzymes to cross the blood-brain barrier (BBB) remains a significant challenge. We propose AAV-mediated gene expression in the brain endothelium as a strategy to address neurological LSDs through the phenomenon of cross-correction, in which transduced cells secrete enzymes that are endocytosed by non-transduced cells. We sought to express human iduronate-2-sulfatase (hIDS) in the brain endothelium of a mouse model of Mucopolysaccharidosis type II (MPSII, Hunter's syndrome) to enable enzyme secretion into the brain parenchyma. In this disorder, IDS deficiency results in the accumulation of heparan and dermatan sulfate GAGs. We used AAV-BI30, an AAV9-derived capsid that has an enhanced *in vivo* tropism specific to the endothelium in the rodent CNS and can transduce human brain vascular endothelial cells *in vitro* more efficiently than AAV9. We show that systemic delivery of AAV-BI30:hIDS restored IDS enzyme activity in the brain, liver, and serum of IDS-KO mice and reduced GAG accumulation in the brain (Fig. 1). The cross-correction was not observed when using the AAV-BI30 vector expressing a non-secreting version of hIDS. These findings highlight that targeting the CNS endothelium is a promising approach for delivering enzymes across the BBB and restoring lysosomal metabolism.

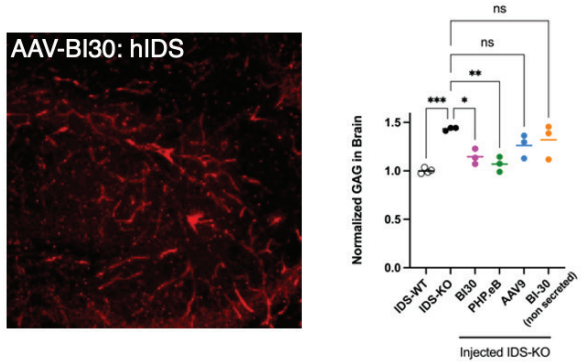

Figure 1. Systemic administration of AAV-BI30:hIDS leads to reliable expression of hIDS by the brain vasculature and corrects pathological glycosaminoglycan accumulation in IDS-deficient mice. (***Left***) Adult C57BL/6J mice received intravenous administration of 1 x 10^{11} vg/animal AAV-BI30:hIDS. Consistent with the known AAV-BI30 tropism, hIDS was expressed primarily in endothelial cells in the CNS. (***Right***) Glycosaminoglycan (GAG) content in the brains of 8- to 11-week-old wild type (WT) and IDS-knockout (KO) mice was quantified four weeks after intravenous treatment with 3 x 10^{11} vg/animal of therapeutic vectors. The results are normalized to the IDS-WT control. Lines represent the mean.

909 Biodistribution of Engineered ARRDC1-Mediated Microvesicles for Delivery of Intracellular Payloads *In Vivo*

Shu-Lin Liu, Yashodhan Chinchore, Ryan von Kleeck, Carson Semple, Lucy (Yumei) Sun, Pearl Moharil, Qiyu Wang, Komal Vyas, Kristin Luther, Adam Francoeur, Leah Gens, Alysia Bryant, Joseph Nabhan

Vesigen Therapeutics, Cambridge, MA

Limited avenues are currently presented for the safe and specific *in vivo* delivery of large and potentially therapeutic molecules. We engineered a class of human vesicles called ARMMs (ARRDC1 Mediated Microvesicles) to enable delivery of a wide range of payloads including RNA, proteins and gene editing protein/RNA complexes (Cas9/gRNA). To determine the identity of cell types amenable to transfection by ARMMs *in vivo*, we initially injected ARMMs loaded with GFP or mCherry as fluorescent reporter payloads through multiple routes of administration: Intravenous (IV), intranasal (IN), intratracheal (IT) or subretinal (SR) injection. To demonstrate the functional delivery of payloads by ARMMs *in vivo* we utilized Cre reporter mouse models. We injected ARMMs loaded with Cre protein in appropriate reporter mouse lines, Ai9 and Ai14, through multiple routes of administration as previously noted. Delivery of a reporter protein or Cre, leading to activation of tdTomato expression in target cells, was utilized to identify specific tissues and cell types that can be efficiently transfected by ARMMs. We use a combination of immunofluorescence staining and flow cytometry to generate the biodistribution profile of engineered ARMMs *vis-à-vis* the route of administration. This dataset enables the articulation of high confidence in rationale therapeutic strategies enabled by ARMMs as a delivery vehicle.

910 Heat Stress on Baculovirus Infected SF+ Cells May Lead to Product Loss

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A platform process has been developed for production of adeno-associated virus (AAV) product using baculovirus infected SF+ cells. This study highlights the impact of bioreactor temperature on subsequent filtration efficiency. In this study, the production of AAV was done at three different temperatures in the bioreactor. The results showed visual differences as well as turbidity differences among temperature conditions. Extra bands were seen on protein gels on the higher temperature conditions while the intensity of other bands noticeably decreased. It was concluded that temperature post infection can play a significant impact on titer and product characteristics.

911 Gene Therapy Targeting Sphingolipid Metabolism for the Treatment of Pulmonary Hypertension

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Up-regulation of ceramides in pulmonary hypertension (PH), contributes to perturbations in sphingolipid homeostasis and the transition of cells to a senescence state. We assessed the safety, feasibility, and efficiency of acid ceramidase gene transfer in a rodent PH model. A model of PH was created by the combination of left pneumonectomy and injection of Sugen toxin. MRI and right heart catheterization confirmed the development of PH. Rats were subjected to intratracheal administration of a synthetic adeno-associated viral vector (Anc80L65) carrying the acid ceramidase gene (Anc80L65.AC), an empty capsid vector, or saline. Therapeutic efficacy was evaluated 8 weeks after gene delivery. Hemodynamic assessment four weeks after the PH model creation demonstrated an increase in the mean pulmonary artery pressure, which was consistent with the definition of PH. We documented a significant increase in pulmonary vascular resistance in the saline-treated and empty capsid groups, but not in rats receiving Anc80L65.AC. Histology confirmed the right ventricular hypertrophy only in PH, saline-treated, and Anc80.Null groups. AC treatment significantly decreased the fibrosis. There was a statistically significant difference between the percentage of remodeled vessels between the PH and AC groups. Immunohistochemical analysis of small pulmonary arteries demonstrated vascular thickening and remodeling at eight weeks. Statistically significant differences were presented in the percentage of occluded vessels and medial wall thickness between Anc80. Null, and Anc80.AC groups. After Anc80L65.AC. delivery, a significant decrease in pro-inflammatory factors, interleukins, and senescence markers was observed. Gene delivery of acid ceramidase provided tropism to pulmonary tissue and ameliorated vascular remodeling with right ventricular dysfunction in pulmonary arterial hypertension. Here we demonstrated the feasibility, efficiency, and safety of airway distribution and transduction of aerosolized Anc80L65. AC is a gene therapy for the pulmonary vessels. In this study, AC overexpression improved cardiopulmonary hemodynamics and RV parameters in rodents' PH models. Sphingolipids metabolism pathways represent the main frontiers in the study of molecular cardiology. Gene transfer via intra-tracheal administration of AC can balance the physiological level of ceramide, provide tropism to the pulmonary tissue, and ameliorate vascular remodeling. Combining Anc80L65 technology with AC gene cassette offers a disease-modifying and robust solution for PH patients.

912 Characterization of Preclinical Models for Oncolytic Adenoviruses

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Oncolytic adenoviruses (OAds) are powerful tools in cancer treatment as they can selectively infect and lyse targeted cancer cells sparing the normal tissues. Importantly, OAds are able to activate antitumor immune responses through the induction of immunogenic cell death and interference with the immunosuppressive tumor microenvironment. Moreover, upon intravenous delivery, OAds can potentially reach distant metastatic sites allowing to perform the systemic treatment. However, the clinical development of OAds has been hampered by the lack of reliable animal models that can predict the therapeutic and immunomodulatory responses of OAds, especially those injected systemically. For the comprehensive evaluation of systemically administrated OAds, the animal models should have the following characteristics: 1) they should support OAd replication, 2) they should be immunocompetent to represent OAd-induced immune response. Third, the interaction of OAds with various blood cells and factors should resemble that in humans. However, the rodent models currently used for OAds assessment do not meet these criteria. Mice do not support replication of all human adenoviruses (Ads) which results in the lack of functional progenies. Moreover, none of the existing rodent models (including mice, Syrian hamsters, and cotton rats) are suitable for the evaluation of species B Ads, (i.e., Ad3, Ad11, Ad 35) since rodent cells lack CD46 and human desmoglein-2, DSG2, receptors. The same is true for the broadly used chimeric OAd vectors retargeted to species B Ads receptors (i.e., Ad5/3, Ad5/35). In this study, in response to the recent NIH Reproducibility Initiative, we aim to seek new and characterize the existing preclinical models for OAds evaluation. We have recently demonstrated that porcine cells and tissues can efficiently support the replication of both species B and C OAds at a level similar to human tissues. Here, we compare these findings with other models evaluating the binding, transduction, and replication efficacy of group B and C OAds (Ad5, Ad6, Ad3, Ad5/3) in various mammalian models including mice, hamsters, rats, dogs, and non-human primates. To better evaluate the OAds performance after systemic delivery, we have investigated its interaction with blood factor X (FX). Adding porcine FX to CHO cells (Chinese hamster ovary cells known to lack CAR, DSG2, and CD46 receptors) significantly increased the binding ability of both Ad5 and Ad5/3 vectors at levels similar to human FX. Additionally, we are analyzing the hemagglutination ability of Ad types toward red blood cells (RBCs) of different mammals. Our preliminary data suggest that porcine and mice RBCs do not

hemagglutinate in the presence of Ad5 and Ad5/3, while human RBCs demonstrate the ability to agglutinate in the presence of Ad5 but not Ad5/3. We anticipate these studies will provide important knowledge to assess the therapeutic, immunomodulatory, and safety profiles of OAds.

913 Small Molecule Epigenetic Modulators for Ex Vivo Expansion of Human Hematopoietic Stem Cells

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Expansion of hematopoietic stem cells (HSCs) for therapeutic purposes has been a "holy grail" in the gene therapy field for many years. Different expansion protocols have been tested in clinical trials, but only moderate *ex vivo* expansion of HSCs has been achieved. Here, we aimed to find optimal protocols for *ex vivo* expansion of HSCs under serum-free conditions using epigenetic regulators such as CPI203 and Quisinostat. We used human cord blood HSPCs ($CD34^+$) as a source of HSC.Since $CD34^+$ is a heterogenous population, advanced flow-cytometry was used to characterize and compare the composition of stem cells and multipotent progenitors. Both *ex vivo* and *in vivo* results suggest a positive role of CPI203 and Quisinostat on *ex vivo* expansion of HSCs and negative effect of IL3 during *ex vivo* culture of human cord blood HSCs. Clinical implementation of these findings is crucial to eventually boost HSC based gene- and cell-based therapies.

914 Chemically Defined and High-Yield AAV Vector Production Medium for Suspension HEK293 Based Cell Lines

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Introduction: Recombinant adeno-associated virus vectors (rAAV) have been widely used as vehicles to deliver gene of interest for basic research or clinical and pre-clinical study of gene therapy. One of the issues to use rAAV vectors for gene therapy is large scale manufacturing. In this regard, suspension culture system using HEK293 based cell lines is highly attractive due to its scalability. Here, we describe our novel high-yield rAAV vector production medium for suspension culture which is chemically defined, serum-free, protein-free and animal origin-free. **Methods:** Viral Production Cells (VPC), Viral Production Cells 2.0 (VPC2) (Thermo Fisher Scientific), and FreeStyle™ 293-F Cells(GIBCO) were used for evaluation of the high-yield rAAV vector production medium. Cell proliferation capacity, cell survival and cell density were tested using the cells conditioned with the medium prior to use in the experiments. The rAAVs produced by transient transfection were extracted by AAV Extraction Solution (AAVpro® Cell & Sup. Purification Kit Maxi,Takara Bio), and the viral genomic copy numbers were measured by the quantitative PCR using AAVpro® Titration Kit (for Real Time PCR) Ver.2 (Takara Bio). **Results:** The newly developed high-yield rAAV vector production medium could support cell proliferation of 3 different HEK293 based suspension cell lines. The cell proliferation capacities cultured in the novel medium were comparable to those cultured in the commercially available medium. The cell densities were reached to 8 - 10 x 10^6 cells/ml when the cells were kept in culture for 7 days. In addition, more than 1 x 10^{13} viral genomic copy number/l of rAAV2 were obtained from the cells cultured in the novel medium and the rAAV production efficiencies were 20 - 50 % higher than those in the commercially available medium. **Discussion:** These results show that our novel high-yield rAAV vector production medium can be optimal and useful for large scale manufacturing of rAAV in suspension culture system.

915 rAAV-Delivered Hepatocyte-Specific Expression of miR-375 Protects Against the Acetaminophen-Induced Acute Liver Failure in Mice

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Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in many countries, including the United States. The high incidence of mortality associated with APAP-ALF can be attributed to its rapid onset. N-acetylcysteine (NAC) treatment, though effective, is time limited. Beyond ten hours after an over-dose, the liver cannot be rescued. Many studies have shown that various microRNAs (miR) can serve as potential biomarkers or therapeutic targets. Among these microRNAs, miR-375 levels correlate with the severity of APAP-challenge significantly in clinical samples, which makes it an appealing target for mitigating APAP-ALF. Using AAV8 capsid and Thyroxine Binding Globulin (TBG) promoter, miR375 was expressed in hepatocytes specifically. When challenged with APAP, ALF could be completely rescued in the miR-375 treated mice compared with control group. Liver APAP metabolites were measured with LC/MS-MS, increased levels of liver-protecting Glutathione (GSH) before APAP treatment and decreased cytotoxic byproduct NAPQI (N-acetyl-p-benzoquinone imine) protein adducts after APAP challenge were observed in miR-375 expressing liver. RNA-seq analysis identified three novel targets, Slc16a2, Acls5 and Cyb5b that were downregulated by miR-375. *In vivo* silencing using artificial miRNA (amiR) against these three genes via AAV8 could protect the liver from APAP-ALF. In addition, Cyp2E1, a key enzyme which was involved in the APAP metabolism into NAPQI, was downregulated by miR-375. To investigate if Cyp2E1 inhibition by miR-375 was required for the liver protection, we used a lower dose of miR-375 without changing the level of Cyp2E1, the liver could still be protected from APAP-ALF to a less degree. Partial/Incomplete protection of APAP-ALF could also be achieved by lowering Cyp2E1 to the level of which miR-375 treatment induced using fine-tuned dose of amiR against Cyp2E1. These results implied the protection by miR-375 was not fully Cyp2E1 dependent and other factors were involved. RNA-seq experiment using livers treated with miR-375 and amiRs against Slc16a2, Acls5 and Cyb5b was carried out. It revealed that the inhibition of Cyp2E1 was accompanied by upregulation of other key enzymes such as Gstm2, Gstm3 and Sult2A7, which were involved in APAP detoxification. Over-expression studies showed that Gstm3 could protect the liver from APAP-ALF in WT

mice. Cyp2E1 heterozygous mice has been acquired and TBG-driven Gstm3 was delivered into the liver with AAV8, the results showed that APAP-ALF was well protected. Taken together, decreasing Cyp2E1 and the production of NAPQI along with increasing Gstm3 and liver glutathione were the two major pathways that miR-375 achieved the liver protection against APAP-ALF. We are exploring if miR-375 can serve as an alternative or better treatment than NAC in acetaminophen overdose and other liver diseases. **J.L. and Y.W. are co-first authors; G.G. and J.X. are co-corresponding authors.**

916 VP1 Peptide Insertions Block Anti-AAV Antibody Binding and Neutralization

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Adeno-associated viral (AAV) vectors have been used in gene therapy clinical trials for almost two decades. First generation constructs were based on naturally occurring serotypes isolated from human or non-human sources. These naturally occurring serotypes rarely possessed all the desired attributes and often lacked the biodistribution necessary to target extra-hepatic tissues. Second generation capsids were developed to have more desirable attributes by engineering the capsid sequence. Capsid engineering by insertion of short peptides at solution exposed hyper-variable region (HVR) has provided considerable benefit in targeting multiple tissues by crossing anatomical barriers. Here, we evaluated binding and neutralization of four AAV9 engineered peptide inserted capsids (9P801, EPIC1-2, and AAV-PHP.B) by two monoclonal anti-AAV9 antibodies, ADK9 and pMAb9; a commercially available antibody and a proprietary antibody, respectively. A cell-based transduction inhibition assay or an ELISA-based assay was used to quantify neutralizing and binding antibodies, respectively. While both antibodies neutralized AAV9 capsid and prevented transduction (IC50 59.33 and 68.22 ng/mL), they had no activity against two molecularly distant AAVs (AAV3B and AAV6). Interestingly, both monoclonal antibodies also did not neutralize the four engineered capsids which differed from AAV9 only by peptide insertion in HVR VIII. Binding antibody assays confirmed these findings with both monoclonal antibodies only binding AAV9 and not the engineered capsids. Collectively, these results suggest the site of peptide insertion overlaps an antigenic site recognized by these monoclonal antibodies. Next, we compared the seroprevalence of NAb against one of the novel capsids (9P801) in healthy human donors (N=100). 9P801 seroprevalence was lower compared to AAV9 (30% vs 35%, 9P801 vs AAV9). Even among positive donors, the magnitude of neutralization was lower with nearly twice as many human donors with high titer AAV9 NAb compared to 9P801. Also, the average 9P801 NAb titers were lower by at least 38% when compared to AAV9. Similarly, in cynomolgus macaques twice as many animals had higher titer AAV9 NAbs with average 9P801 titers lower by 62%. Overall, our studies demonstrate that capsids with peptide insertions in HVR loop that overlap a potential antigenic site are less effectively bound and neutralized by antibodies that may allow dosing of patients with low level NAb over the parental capsid, while also increasing their overall safety and efficacy following systemic administration.

917 High-Throughput Genotype and Functional Analysis of CRISPR/Cas9 Edited Hematopoietic Stem and Progenitor Cells Using Single-Cell Genotyping to Transcriptome (GoT)

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Sickle cell disease (SCD) is an inherited disorder caused by a single nucleotide substitution in the beta-globin gene (*HBB*), replacing glutamic acid with valine at the sixth residue. CRISPR/Cas9 gene editing has been proven as an efficient approach to correcting the pathogenic sickle mutation in *HBB* via the homology-directed repair (HDR) pathway after delivery of Cas9/gRNA ribonucleoprotein (RNP) complex and corrective donor template. However, genome editing-induced DNA double-stranded breaks (DSBs) can result in complex gene modifications, which could lead to detrimental phenotypes. We clonally genotyped 80 erythroid colonies differentiated from patient hematopoietic stem and progenitor cells (HSPCs) treated with R-66S RNP targeting the sickle mutation on *HBB* and single-stranded oligonucleotide (ssODN) donor. We found that 18.5% of the colonies carried large deletions (LDs), with a very diverse allelic genotype including HDR/LD (8.2%), small insertions or deletions (Indel)/LD (9.0%), and HBD gene conversion/LD (1.0%). The high rates of unintended consequences, including gene ablation, mRNA truncation, misregulation, and deleterious protein products, could significantly reduce the therapeutic effects of gene correction, and cause safety concerns. Therefore, high-throughput genotype and phenotype analysis of single cells is critical to evaluate the safety and efficiency of gene editing-based therapy. We used single-cell genotyping of transcriptome (GoT) analysis, which can track the transcript of an edited gene and its transcriptome using a shared barcode from single-cell RNA sequencing (scRNA-seq), providing a method to directly link functional consequences with a specific genotype in high throughputs. Using scRNA-seq GoT, we analyzed R-66S RNP treated, R-66S RNP+ssODN treated, and untreated SCD HSPCs at 0 days, 4 days, and 10 days after erythroid differentiation. The *HBB* mRNA-tagged cell-specific barcodes from edited and unedited cells were amplified and sequenced on MiSeq. We developed a bioinformatics pipeline to accurately identify the genotypes in edited cells with major functions such as unique molecular identifiers (UMI) consolidation and editing outcomes quantification. The transcriptome libraries were sequenced on Next-seq and were analyzed using the Seurat R package. We conducted a global analysis of the gene expression and generated cell clusters using the uniform manifold approximation and projection (UMAP) method. The genotyping results were then mapped to corresponding cells in UMAP cell plots. On 10 days post differentiation, the GoT analysis of 2,111 RNP-treated cells detected at least 289 unique genotypes, categorizing into 18 groups: homozygous (homo) SCD, homo HBD, homo LD, homo SI In, homo SI Out, heterozygous SI In/SI In, SI Out/SI Out, SI In/SI Out, SCD/SI In, SCD/SI Out, LD/SCD, LD/SI In, LD/SI Out, LI/SI Out, HBD/SCD, HBD/SI In, HBD/SI Out, and HBD/LD,

in which we defined LD as large deletion, LI as large insertion, SI In as in-frame small indel and SI Out as out-of-frame small indel. RNP-treated SCD HSPCs formed a unique cluster of cells displaying impaired erythroid maturation phenotype with a high abundance of biallelic *HBB* knockout genotype compared to the untreated sample. In summary, our GoT analysis revealed strikingly high genotypic heterogeneity in gene-edited cells at a single-cell resolution and directly linked gene editing introduced HBB genotypes to varying degrees of erythroid maturation phenotype. We will further dissect the genotype and phenotype of gene-edited cells to derive novel insights into genotypes that led to impaired phenotypes and their molecular pathway, which is critical for the accurate evaluation of biological/clinical consequences of gene editing for safe clinical translation.

918 Characterization of Exo-AAVs Produced from AAV8, 9 and 10 Serotypes

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Recombinant adeno-associated viruses (rAAVs) are currently being explored as treatments of several nervous system disorders. Modifications of rAAVs are being developed that help increase transduction efficiency, targeting of specific cell types (e.g., neurons or glia) and to cross the blood-brain-barrier after systemic infusions. Additionally, there is great interest in finding means of the rAAV to evade the immune system to avoid the production of neutralizing antibodies that will limit the rAAV's ability to be used multiple times. Recently it has been discovered that rAAV can exist in an exosomal associated form. These so called exo-AAV formulations have several interesting properties that can be leveraged in treating CNS disorders. For example, the exosomal envelope appears to prevent the immune system from recognizing the rAAV with little to no neutralizing antibody production to exo-AAV formulations. Further, the presence of the exosomal envelope allows us to leverage targeting strategies to create formulations of exo-AAV that are permeable to the blood brain barrier. Alternatively, targeting strategies can be used to limit transduction to specific cell types within the brain, which is critical in treating neurological disorders whose etiology is based in a specific subset of cells (e.g., dopamine neurons in Parkinson's Disease). The rise in use of rAAVs in treating CNS disorders over the past decade comes in large part from the discovery of AAV serotypes that readily transduce nervous tissue with great efficiency, including AAV8, 9 and 10. Therefore, as a critical first step in exploring the use of exo-AAVs in CNS applications, we have determined the packaging efficiency of exo-AAVs based on common rAAV serotypes used in CNS applications. In this study we have isolated exo-AAV preparations from different rAAV serotpyes using sequential centrifugation of HEK-293 cell cultures actively producing standard pseudotyped rAAVs. The culture media was centrifuged at 1000 x g followed by 20,000 x g and then 100,000 x g to isolate the exosomal fractions. The preparations were then titered, nanaoparticle sizes were analysed and the viruses were tested via transduction of various cell types. We have determined that rAAV2/8, 2/9, and 2/10 all package efficiently in exo-AAV formulations where they are produced with high titer and retain good transduction efficiency. We discuss modifications to the packaging procedure that may help optimize the production of exo-AAVs based on different serotypes.

919 Direct Comparison of Muscle Specific Promoters in Proliferating and Differentiated Myogenic Cells

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The heterogeneity of muscular tissues complicates the development of gene therapy that would be restricted to one or equally effective in different muscle type. A promoter for muscle gene therapy should exhibit limited activity in non-targeted tissue, while conferring specific, long-term sustained and physiological expression in the desired muscle types. Several muscle specific promotors have been identified and further optimized. Several of them have proven efficiency and some are widely used throughout the research community. However, a direct comparison between different muscle specific promotors is hard to find. Here we present a direct comparison of muscle specific Desmin and MCK-based chimeric MHCK7 promoter as well as microRNA206 and Calpain3 promoter. Desmin and MHCK7 promoters are said to be highly active in murine and human skeletal muscle and in the heart, whereas microRNA206 and Calpain3 promoter where described provide gene expression mainly restricted to skeletal myocytes. Reporter gene expression mediated by the different promoters was analysed by plasmid transfection using optimized protocols ensuring similar transfection efficiencies human and murine skeletal myoblasts and cardiomyocyte precursors. Since both, the activity of human promoters and the complexity of protein related physiological processes may differ in the animal model compared to the human organism, we present an *in vitro* model based on electrical pulse stimulation (EPS) to provoke sarcomere formation in 2D cell culture for quantification of promoter activity in far differentiated mouse and human myotubes. We found that Desmin and MHCK7 promoters showed stronger reporter gene expression levels in proliferating and differentiated myogenic cell lines than miR206 and CAPN3 promoter. However, Desmin and MHCK7 promoters mediated gene expression also cardiac cells whereas miR206 and CAPN3 promoter expression was more restricted to skeletal muscle. Our study provides insights helping to choose optimal muscle specific promotors for an intended treatment approach with regard to muscle tissue specificity, expression strength and duration.

920 Optogenetic Modulation of the Spine for Pain Inhibition

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Introduction: Pain is a nearly universal experience with evolutionary value as a warning system for injury and illness. However, it can also be so pervasive and intense that it becomes a pathological state that impedes physical, psychological, and social function. There are many neurological structures involved in the pain experience which detect, communicate, modulate, and process pain information. The spinal cord and associated structures such as the dorsal root ganglia (DRG) communicate pain information from the peripheral nociceptors. Here, we present a red light-

activated combinatorial gene therapy approach for cell-selective modulation of neuronal circuitry in spine for pain inhibition. **Methods:** Multi-Characteristic Opsin (MCO) having high red-light sensitivity allows deeper tissue penetration (due to low scattering and absorption) while minimizing photo-toxicity. For genetically targeting the expression of MCO in inhibitory (GABAergic) neurons, Glutamic Acid Decarboxylase (GAD) promoter was used. A wireless Optogenetic Pain Modulator (OPM) device was developed to provide optogenetic stimulation with red (630 nm) light in a controlled and programmable manner through the dermis and other overlying tissues. We tested the efficacy of red light in pain modulation using acute (e.g. formalin assay, hind paw injection) and chronic (sciatic nerve constriction, SNC) pain models in mice. We measured reflexive pain responses with and without optogenetic stimulation. Pain scoring in the acute pain model was quantified by measuring the time spent performing typical pain expressions/coping actions within a 60 second span. In the chronic pain model, treatment efficacy was measured by comparing the force needed to cause paw withdrawal (an indication of sensitivity to potential mechanical pain) after chronic injury (which increases sensitivity and decreases the needed force) with and without red light stimulation. **Results:** Red light stimulation of MCO-expressing inhibitory neurons of the spine reduced both acute and chronic pain reflexive responses. Pain responses to formalin injection were significantly reduced in the second phase of the formalin challenge (which is dominated by inflammatory pain sensation). Red light stimulation also increased the threshold for mechanical allodynia in SNC mice. **Conclusion:** Our results support the hypothesis that transdermal red-light stimulation of MCO-sensitized inhibitory neurons of the spine can effectively reduce both acute and chronic pain. The use of a highly sensitive light-actuator (MCO) and the properties of the wireless-controlled OPM device allows a highly customizable cell-specific transdermal red-light stimulation for effective pain modulation with a low power burden.

921 A High-Throughput Small Molecule Screen Identifies Targets That Increase AAV9 Production in Suspension HEK293 Cells

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The use of recombinant AAV as a vector for gene delivery is widespread, with over 900 pre-clinical and clinical programs underway. However, inefficient manufacturing methods result in high costs, limiting the availability of gene therapies. In this study we describe a high-throughput small molecule screening strategy to identify compounds that increase the capacity of cells to produce AAV9. We used the ATLAS (Arrayed Targeted Library for AAV Screening) platform to perform the primary screen using a library of over 3000 small molecules. Targets identified included transmembrane proteins, DNA repair proteins, cell-cycle regulators, and epigenetic modulators. The top 70 performing compounds were re-evaluated in a dose-response manner on our proprietary HEK293 cell line (AC001.230). After a series of studies in small and large-scale shake flasks, we identified a novel compound (SM-016) that increased rAAV9 production in a robust and dose-dependent manner. We have confirmed these findings using capsid titer and vector genome quantification using two reporter constructs. Evaluation of SM-016 is ongoing to apply these findings for production of FDA-approved therapeutic transgenes with multiple AAV serotypes and in a large-scale bioreactor system.

922 Biological Pacemaker Activity by AAV9-TBX18 Induced Somatic Reprogramming

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Background: Sinoatrial node (SAN) dysfunction is a clinical disorder characterized by the inability of the SAN to maintain appropriate heart rates at baseline and/or during stress. Patients with this condition often need the implantation of an electronic pacemaker. Although it is the current standard of care, devices can have limitations and complications such as lack of chronotropic response, generator or leads malfunction, and infections. Creation of a biological pacemaker has been shown by gene therapy using adenovirus expressing the human transcription factor TBX18. However, adenoviruses are immunogenic, limiting the duration of transgene expression. **Aim:** We sought to induce somatic reprogramming *in vivo* and create a biological pacemaker using Adeno-associated virus serotype 9 (AAV9) expressing human TBX18. **Methods:** AAV9 containing GFP (control) or TBX18 transgenes were directly injected into the myocardium of eight-week-old male Sprague Dawley rats at low (1x109 GC), mid (1x1010 GC) and high (1x1011 GC) doses. Six weeks after intramyocardial injections, we induced atrioventricular (AV) block *in vivo* using adenosine (10 mg/kg, i.v.) while the rats were monitored by a 6-lead electrocardiogram. Hearts were harvested for optical mapping, histology, and mRNA analyses. **Results:** No electrophysiological abnormalities were noted in either of the groups at baseline. After AV block induction using adenosine, only rats injected with AAV9-TBX18 mid-dose had increased heart rate (111±5.5 vs GFP: 87±10.6 bpm, 27%, p=0.04) compared to AAV9-GFP. Further analysis of the mid-dose groups revealed no histological changes in the injection or remote sites when we compared the GFP and TBX18 groups. We quantified ectopic beats in the mid-dose injected rats and detected a higher number of ventricular ectopic beats in the AAV9-TBX18 (14±7.2 vs GFP: 2±2.0 bpm, p=0.04) group than AAV9-GFP. To verify that the origin of the ectopic beats was indeed coming from the injection site, we performed a 6-lead EKG analysis to obtain the QRS axis in the frontal plane. This revealed that the ventricular ectopic beats from the AAV9-TBX18-injected group originated from the injection site. In addition, we performed optical mapping in isolated hearts from AAV9-GFP and -TBX18 groups after *ex vivo* induction of AV block. Accordingly, activation maps confirmed that the injection site originated those beats in TBX18-injected rats only. Successful reprogramming of adult ventricular myocytes into sinoatrial node-like cells was confirmed by transcriptional profiles with increased expression of TBX18 (2.2±0.5 FC relative to GFP, p<0.0001) and Hcn2 (25.2±1.1 FC relative to GFP, p=0.02), and reduced Gja1(-10.8±0.7 FC relative to GFP, p=0.005), Scn5a (-1.1±0.8 FC relative to GFP, p=0.01) and Nkx2.5 (-2.0±0.6 FC relative to GFP, p=0.0004). **Conclusion:** Collectively, these data indicate that the AAV-based somatic reprogramming creates sustained biological pacemaker activity in rats with AV block. Long-term studies are needed to characterize the long-term effects of sustained TBX18 expression.

924 Precise Measurement of Vector Copy Number and Transduction Efficiency at Single-Cell Resolution for Cell and Gene Therapy Development

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Cell and gene therapies are transformative solutions for patients with inherited and acquired diseases for which existing interventions are ineffective. The therapies target diseases at the cell level and modify or replace genes of specific sets of cells to alter their functionality. Such therapies usually rely on viral transduction or non-viral vector gene transfer techniques to introduce transgenes into host cells. The accurate measurement of gene transfer is critical to the development of therapeutic agents, which directly contributes to their safety and clinical efficacy. Currently, methods for the quantitative measurement of gene transfer lack the resolution and representation to truly reflect sample composition and either report a population bulk average or require labor intensive and time-consuming clonal isolation which can take weeks. Here, using a microfluidic, emulsion based single-cell DNA sequencing platform and a highly multiplexed amplicon panel targeting both vector sequence and human genome, we demonstrate a novel methodology that provides precise and accurate measurements of vector copy number (VCN) and transduction efficiency at single-cell resolution for cell and gene therapy development. A set of well characterized clonal cell lines with a range of known vector copy numbers validated by orthogonal methods was used for assay development. We show that single-cell DNA sequencing identifies transduced versus non-transduced cells with exceptional sensitivity (99.9%) and specificity (99.6%) with high precision. In the same assay output, populational VCN can be measured with high correlation (R squared value 0.99) compared to orthogonal ddPCR values. Due to the simultaneous measurement of transduction % and populational VCN, the assay also provides average VCN per transduced cells from the same data set. Finally, besides average VCN, the distribution of VCN at single-cell resolution can be characterized. The single-cell level vector copy number and transduction assay provides exceptional resolution and valuable insights for those developing more effective and safe therapeutic agents.

925 Dose Ranging Pre-Clinical Studies of Systemic AAV9 with Codon-Optimized Reduced Size ATP7A (cors-ATP7A) Plus Subcutaneous Copper Histidinate in a Menkes Disease Model

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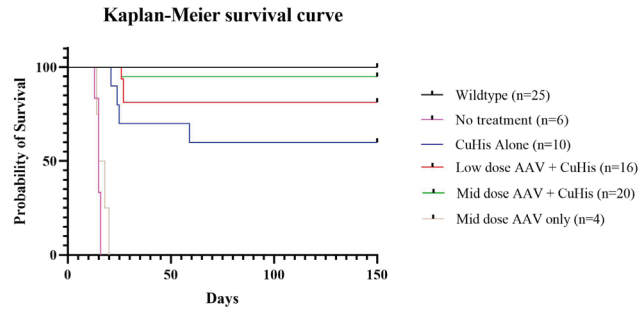
Menkes Disease (MD) is an infantile onset, X-linked recessive disorder of copper transport characterized by rapidly progressive neurodegeneration, seizures, and failure to thrive caused by mutations in *ATP7A*, a copper transporter gene. The *mottled-brindled* mouse model (*mo-br*) harbors a spontaneously arisen deletion in *Atp7a*, the murine homolog, and recapitulates salient features of MD including low serum copper levels, severe neurodegeneration, and early death (postnatal day 13). We previously documented 23% & 53% rescue of this model with CSF-directed AAV5- or AAV9- mediated transfer of a reduced size (rs) version of human *ATP7A*, in combination with subcutaneous copper. Following a CBER/FDA pre-IND meeting, we completed dose-ranging pre-clinical studies of intravenous AAV9 delivering a codon-optimized, reduced size version of *ATP7A* (corsATP7A) plus 30μg clinical grade CuHis by subcutaneous injection. AAV biodistribution in a 12-day wildtype and *mo-br* mouse revealed efficient transduction in various organs except the renal medulla, reflecting inability of AAV particles to effectively traverse renal glomeruli and reach the distal nephron. Safety, efficacy, and toxicological studies identified two doses, $2.6x10^{12}$ and $2.6x10^{13}$ vg/kg body weight + CuHis as highly promising, with combined long-term survival of 89% (n=36) versus 60% in mice receiving CuHis alone (n=10) and 0% for AAV9-corsATP7A alone (Fig. 1). Overall outcomes (serum and brain copper, brain neurochemical ratios, growth, neurobehavior) appear superior in the $2.6x10^{13}$ vg/kg + 30μg CuHis cohort survivors (n=19). Administration of a higher dose, $2.6x10^{14}$ vg/kg, was initially well-tolerated, but sudden death occurred in 5 of 6 animals after CuHis administration on P4 or P5. Postmorten analyses showed no evidence of brain, or liver toxicity however acute renal tubular necrosis and high renal copper levels were noted and considered the cause of death.



While prior studies in human subjects have shown that early intervention with CuHis alone improves survival & clinical outcomes, the overall burden of disease remains considerable. Based on the multi-tasking character of *ATP7A*, Menkes disease infants identified prior to symptoms would appear to benefit from viral gene therapy that provides working copies of ATP7A. Our IND-generating studies in the *mo-br* mouse represent important steps toward a future pilot clinical trial for individuals with this illness. In combination with newborn screening for ATP7A-related disorders, the potential impact of our results on clinical practice is high since the largest barriers to good health for patients with Menkes disease would be circumvented.

926 Targeted Gene Insertion of *Factor 9* as a Potential Durable Treatment for Hemophilia B

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CRISPR/Cas9-mediated genome editing is an emerging approach to treat human diseases by addressing their underlying genetic cause. We have developed CRISPR-based therapeutic platforms to either reduce or restore expression of genes involved in human disease. One such platform is an *in vivo* CRISPR-based gene replacement approach, which involves the targeted insertion of a promoterless expression cassette into the *Albumin* locus of liver hepatocytes using a lipid nanoparticle (LNP) and adeno-associated virus (AAV) delivery system to allow robust and durable therapeutic protein expression from the liver. We have applied this approach to the treatment of hemophilia B, an X-linked genetic disorder characterized by reduced coagulation Factor IX (FIX) expression and impaired coagulation pathway activity. Here, we describe our CRISPR/Cas9-based targeted gene insertion platform technology and highlight how this approach provides important advantages over AAV episome-based therapies. In particular, we demonstrate that expression of FIX via insertion is durable and stable in multiple models of rapid liver growth. To aid with the clinical translation of our *Factor 9* gene insertion program, we established the optimal timing and sequence of the AAV and LNP dosing regimen in multiple model systems. Our development candidate demonstrates robust, dose-responsive human FIX expression in both rodents and primates. We have established that liver insertion-derived FIX protein is functionally indistinguishable from purified human FIX, and treatment with our development candidates rescues hemostasis in hemophilia B mice. We are developing a suite of molecular tools to further monitor and characterize insertion products at the level of DNA, RNA, and protein. Our *Factor 9* gene insertion program may represent a novel treatment for patients with hemophilia B and highlights the potential of our liver gene insertion platform technology to address a variety of indications that would benefit from durable gene replacement therapy.

927 Development and Optimization of a Feeder Cell Free TIL Therapy Platform

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Tumor-infiltrating lymphocyte (TIL) Therapy, a type of adoptive cellular therapy, has garnered increasing attention across the cell and gene therapy industry over the last 40 years. Typically performed by harvesting infiltrated lymphocytes from tumors, co-culturing with feeder cell lines, amplifying or modifying, and infusing back to patients for the successful treatment of solid tumors, many groups from both academia and industry have made TIL therapy variations a focus of research and clinical trials. Unfortunately, despite encouraging results in early studies, TIL therapies continue to face challenges such as side effects coming from high-dose IL2 requirements, TIL exhaustion after long-time culturing in vitro, lack of persistence in patients, and various feeder cell challenges. As an industry leading Contract Testing Development and Manufacturing Organization (CTDMO), WuXi Advanced Therapies (WuXi ATU) is developing a feeder cell-free TIL platform aimed at enabling ongoing research and pre-clinical and clinical phase TIL based therapies. The WuXi ATU TIL Platform is a continuously developing program that focuses efforts on leveraging automated joint enzymatic and physical dissociation of tumor tissues, combined with lower dose cytokines and optimized growth parameters, media, and activators to stimulate growth of potent and healthy TIL. Originally, the platform was developed by using dissociated tumor cells (DTCs) harvested and banked from patients with carcinomas and adenocarcinomas of various origins. We were able to perform design of experiment (DoE) comparison studies to evaluate new available media, activators, and cytokines in comparison with the platform reagents for the process performance and final product quality. After establishing preferred parameters using the DTCs, development was expanded to combine our preferred parameters with fresh tumor tissue obtained from cancer patients. Conclusions: 1)TIL expansion starting from disintegrated tumors rather than from tumor fragments is a viable method for TIL therapy initiation.2)TIL growth in the absence of feeder cells is a viable option for TIL therapies.3)TIL proliferation rates and final product cell quality are impacted by basic media and supplements.4) Media type and human serum albumin (HSA) presence make a significant impact on CD4/CD8 ratio.5)Re-stimulation can help to increase TIL population doublings and yields.6)The activation method impacts CD4/CD8 ratio in the final product. We have demonstrated that lymphocytes obtained from DTCs and those harvested from tumor tissue in cancer patients expand differently in different media; we also demonstrated that the CD4 to CD8 ratios in the final products can vary considerably depending on the culture media, activators, and cytokine concentrations. Based on the results we obtained above, we will further optimize our TIL Cell Therapy Platform for successful manufacturing of cell therapies from cancer patient starting material. Ongoing studies include evaluating additional cytokines in various concentration combinations added at different critical time points, investigating re-stimulation impact, and optimizing isolation methods for purification of T cells at critical stages in the process.

928 Cell Type-Specific Genetic Therapy of Cancer, Virus Infection and Mitochondrial Disease

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Cell type-specific gene delivery which restricts gene transfer to therapy-relevant cells, hence reducing unwanted side effects caused by the ectopic expression of transgenes, remains one of the biggest challenges. To mitigate potential off-target effects, we developed a programmable cell type-specific gene therapy using an RNA *trans*-splicing-based approach for gene replacement therapy or suicide gene therapy that can selectively express therapeutic proteins or suicide signals based on the cell's endogenous pre-mRNA expression profile. Using the liver

as a medical target organ, we developed a hepatocellular carcinoma (HCC)-specific *trans*-splicing based Herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) suicide gene therapy approach. We designed suicide RNAs that can target multiple HCC-related pre-mRNA biomarkers thereby increasing the overall concentration of targetable pre-mRNA. Our latest generation of such multi-targeting constructs exhibited the highest cell death activity even at a GCV concentration of only 0.3 μM. For cell-type specific delivery, the conjugation of dumbbell vectors with $GalNAc_3$ residues enabled targeted delivery into hepatoblastoma-derived human tissue culture cells. To deliver nucleic acids into the mitochondria of human cells, we studied the long non-coding β2.7 RNA of the human cytomegalovirus which was previously reported to colocalise with mitochondrial complex I. Using thermodynamic profiling, we identified four thermodynamically conserved structural subdomains within the β2.7 RNA that are responsible for its mitochondrial targeting activity. A vector comprising eight mitochondrial targeting domains exhibited a three times higher mitochondrial targeting activity as compared with the parental β2.7 RNA. The amalgamation of mitochondrial targeting RNA and *trans*-splicing allows cell type-specific transcription of mRNA which can be delivered into mitochondria for mitochondrial gene therapy. *Trans*-splicing based gene therapy represent a technology that is highly translational offering promising perspectives towards suicide gene therapy of cancer or incurable infections with integrating viruses and may enable cell type-specific mitochondrial gene therapy.

929 Optimizing AAV Transduction with Modulators of DNA Repair Pathways

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Introduction: Modulating DNA repair pathways can be associated with enhanced adeno-associated virus (AAV) gene expression, leading to an interest in using these modulators to enhance AAV delivery *in vivo*. The role of many of these modulators in AAV integration has not been studied in detail, raising concerns about potential genotoxicities. AAV's safety derives partly from its primary persistence as an extrachromosomal episome, but the integration of AAV DNA has the potential to result in genotoxicity. Preclinical studies have identified cases of hepatocellular carcinoma and clonal expansion linked to integrated AAV in animal models. AAV integration has been associated with double-stranded DNA breaks and is mediated by DNA repair pathways, such as non-homologous end joining (NHEJ). As rearrangements and truncations have been identified in integrated AAV genomes, we have been developing methods to assess the effects of DNA repair modulators on AAV integration and rearrangement. **Methods:** We have developed a system for modulating DNA repair pathways during AAV transduction of primary cells and transformed cell lines. After diluting out episomal forms, we measured transgene expression and vector copy numbers (VCNs) to estimate integration frequency. Then, the structure and frequency of AAV integrands in these cells were characterized with ligation-mediated PCR and Illumina paired-end sequencing methods followed by analysis with the custom software pipeline AAVengeR. **Results:** Preliminary results have identified modulators of DNA repair pathways that only modulate early transduction as well as modulators that affect both early transduction and integration. Initial results suggest that increases in transduction in the presence of some modulators may not be entirely due to increased second-strand DNA synthesis and begin to indicate additional mechanisms. **Conclusions**: By developing a better understanding of these associations, we hope to optimize AAV transduction while reducing integration-mediated genotoxicities

930 Characterization of the Muscular and Cardiac Diseases of the DMSXL Mouse Model, a Transgenic Mouse Model for Myotonic Dystrophy Type 1

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Myotonic Dystrophy type 1 (DM1) is an autosomal dominant, progressive, and multi-systemic genetic disorder affecting at least 900,000 individuals worldwide. It is primarily characterized by myotonia, muscular weakness, and muscle atrophy. Other clinical manifestations include cardiac conduction defects, cardio-respiratory problems, cataracts, endocrine dysfunction and frequent neurological manifestations. The disease is caused by unnaturally expanded repeats of CTG trinucleotide in the 3'-untranslated region of the *DMPK* (dystrophia myotonica protein kinase) gene. In DM1 patients, the number of CTG repeats within the mutant *DMPK* allele ranges from 50 to 5000. The number of repeats roughly correlates with disease onset, severity and life expectancy (more repeats correlated with earlier onset, more severe symptoms and shorter lifespan). CTG-containing mutant DMPK transcripts are toxic. They aggregate as nuclear *foci* and impact the expression and function of RNA-binding proteins (such as MBNL1 and CELF1), resulting in spliceopathy of downstream effector genes, which accounts for much of the disease phenotype. Several therapeutic approaches either pharmacological or gene-therapy based, are under investigation to address this unmet medical need. One current limitation for the efficient evaluation and development of therapeutic products is the lack of DM1 animal models that ideally recapitulate the symptoms and the complex pathophysiology of this disease. Among available animal models, one of the most relevant remains the DMSXL mouse model, which carries a 45-kb human genomic fragment including the *DMPK* gene with more than 1200 CTG repeats. The human *DMPK* transgene is under the control of its own promoter and has been shown to have an almost ubiquitous expression. Initial characterization studies demonstrated

that homozygous DMSXL mice display several manifestations of the human DM1 pathology, including growth retardation, muscle defects, cognitive impairments, nuclear *foci,* and splicing abnormalities. After establishing a colony in our own facility, our goal was to define in our hands the most relevant and sensitive readouts that characterize this animal model, especially for its muscular and cardiac diseases, in both genders. Different groups of male and female DMSXL mice and littermate wild-type controls were followed and evaluated at 1 and 4 months of age. Weights, clinical status and blood biomarker concentrations were analyzed. Muscle strength and endurance were measured using grip force test and treadmill test, respectively. Cardiac function was evaluated using 2D-echocardiography analysis, and conventional measurement of electrocardiography parameters before and after challenge with flecainide, a pharmacological stressor of conduction disorders. Finally, after sacrifice, muscle and cardiac tissues were analyzed for levels of expression of human and murine DMPK transcripts, expression of MBNL1 and CELF1, splicing abnormalities, CTG-containing nuclear *foci*, muscle fiber type, muscle fiber size, and global histopathological lesions. Importantly, to avoid bias in the analysis, experiments were done in a blinded fashion. The data obtained in the frame of this study will be presented. They will be of importance to design further preclinical studies for the evaluation of the efficacy and safety of different therapeutic products designed to treat DM1 at the muscular and cardiac levels.

932 RGX-111 Gene Therapy for the Treatment of Severe Mucopolysaccharidosis Type I (MPS I): Interim Analysis of Data from the First in Human Study

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MPS I is a rare, autosomal recessive disease caused by deficiency of alpha-L-iduronidase (IDUA), an enzyme required for the breakdown of lysosomal glycosaminoglycans (GAGs). These GAGs accumulate in tissues of MPS I patients, resulting in characteristic storage lesions and diverse clinical signs and symptoms. In Hurler and Hurler-Scheie MPS I phenotypes, central nervous system (CNS) GAG accumulation leads to hydrocephalus, spinal cord compression, and cognitive impairment. Current disease-modifying therapies for MPS I include hematopoietic stem cell transplant (HSCT) and intravenous enzyme replacement therapy (ERT) with recombinant human IDUA. However, ERT does not treat the CNS manifestations of MPS I, and HSCT can be associated with clinically significant morbidity and mortality. RGX-111, a recombinant adeno-associated virus serotype 9 capsid containing a human IDUA expression cassette (AAV9.CB7.hIDUA), administered to the central nervous system (CNS) may provide a permanent CNS source of secreted IDUA, potentially preventing the progression of cognitive deficits that otherwise occur in MPS I patients. In this phase 1/2, first-in-human, multicenter, open-label, dose escalation trial (NCT03580083), participants with documented evidence of CNS involvement or severe MPS I $\geq$ 4 months of age receive one image-guided RGX-111 injection to the CNS with 104 week follow-up for safety, tolerability, and efficacy. Participants are encouraged to enroll into a long-term, follow-up study for a total of 5 years. Assessments include measurements of cerebrospinal fluid, plasma, and urine biomarkers; cognitive, language, and motor neurodevelopmental scales; and imaging. Subjects have been enrolled at Dose 1 and Dose 2 ($1.0x10^{10}$ and $5.0x10^{10}$ genome copies/gram brain mass, respectively). Interim results of this trial will be presented. Additionally, follow-up will be reported for a severe MPS I child treated at age 21 months utilizing a single-patient, investigator-initiated Investigational New Drug application.

933 Hybrid Dual AAV Vector System with Novel Synthetic Splice Enhancer Elements for Increased Efficiency of Splicing and Gene Expression

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The cargo DNA packaging capacity limitation of recombinant AAV vectors of 4.7kb are overcome by various dual/split gene strategies. Current dual vector strategies have demonstrated superiority in final transgene expression level from the delivery of large therapeutic genes. Dual hybrid vector platforms utilise both ITR mediated and an intronic overlapping element for recombination between the two half-vector genomes for transgene reconstitution. The splice donor and acceptor signals flanking the overlapping sequence thus affect the intron removal, highlighting a critical hurdle for final protein expression. Here, we have developed a synthetic, small sequence containing splice enhancer elements arranged in tandem with high recombination potential to test the hypothesis that dual vector efficiency may be enhanced by efficient splicing. This novel 90 bp synthetic splice enhancer intron (SEIa) was cloned into *LacZ* and *ABCA4* cassettes driven by CMV promoter. SEIa was compared to the previously reported 297 bp AP sequence (OAP). Single vector *LacZ* control and hybrid dual vector genomes containing either SEIa or OAP were packaged in AAV9 capsids for *in vitro* and *in vivo* analysis. Subretinal and intramuscular *LacZ* dual vector injections were administered into 4-week-old C57BL/6 mice at each vector dosage of 2.5x10^9vg/eye and 1x10^10vg/TA muscle. *LacZ* expression was analysed by histochemical staining, 6-8 weeks post injection. Percentage of *LacZ* positive TA muscle fibres from the SEIa vectors (82.9% ±3.8) were significantly (p=0.0004) higher compared to the OAP vectors (48.0% ±6.6). In the retina, *LacZ* expression measured by the diffusion area percentage were significantly (p=0.03) higher in the SEIa group (45.8 ±0.8) compared to the OAP group (40.5 ±1.4). Molecular analysis of splicing efficiency using qPCR and PCR for proportions of spliced and unspliced mRNA demonstrated lesser proportions of unspliced mRNA in the SEIa dual vectors compared to OAP. We further applied this strategy to the 6.8kb gene *ABCA4*, which is mutated in Stargardt's macular degeneration and tested it for *in vivo* functional recovery. Six-month-old *Abca4*$^{-/-}$

animals were injected sub-retinally with AAV9.*ABCA4* dual vectors, 5′ or 3' vector alone or sham. Retinal structural and functional changes of all groups were assessed weekly by fundus and FFA imaging, ERG analysis prior to sacrifice followed by histology 8 weeks post injections. AAV dual vectors (both SEIa and OAP) administered *Abca4*$^{-/-}$ eyes demonstrated significantly reduced lipofuscin deposits (~60-70%) and significantly recovered retinal function on ERG (n=6/group, $p<0.05$) compared to single vector (half gene) and sham controls 2 months post infection. While both OAP and SEIa dual vector retinae had higher levels of Rhodopsin and Opsin expression, the SEIa dual vector treated eyes showed highest levels of Rhodopsin expression compared to control *Abca4*$^{-/-}$ eyes. The synthetic 90 bp splicing enhancer sequence containing AAV dual vectors performed better than the currently existing dual vectors in both retina and muscle. The smaller sequence further increases the overall packaging capacity by an additional 400bp without compromising on recombination efficiency. We demonstrate that the higher transgene expression is partly explained by the enhanced splicing efficiency. Our results in Stargardt disease model validates the application of this new dual vector strategy for diverse diseases with causative, large genes.

934 Identification of Universal Parameters That Play a Critical Role in rAAV Production Using a Transient Two-Plasmid Packaging System in the Adherent HEK293T Cell Line

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Adeno-associated virus (AAV) is the vector of choice for gene therapy due to its relatively good safety profile and persistence. Consequently, the number of AAV clinical trials are expanding rapidly. To meet the increasing demand, challenges in manufacturing of recombinant AAV (rAAV) viral vectors such as low yields and low full:empty capsid ratios in unpurified material need to be tackled. In this work, we studied the effect of critical process parameters that determine the yield and quality of rAAV production. We started from a baseline rAAV production protocol based on the adherent human embryonic kidney (HEK)293T cell line in combination with a two-plasmid transient transfection system in which *rep* and *cap* genes and Ad helper genes are on the same plasmid. As this protocol routinely yields decent yields and full:empty ratios, we considered it a good starting point for identifying the process parameters that lead to superior rAAV production yields. We evaluated the effect of plasmid quantification, confluency at the time of transfection, total plasmid DNA (pDNA) amount, DNA:PEI ratio, and plasmid ratio on vg titer, VP titer and % full for AAV2, AAV5, and AAV9. In addition, we measured the intracellular average copy number of the different plasmids 16h after transfection in HEK293T cells to allow monitoring of the effect of aforementioned parameters, both on transfection efficiency as on AAV production. In an effort to obtain more reproducible transfection conditions, we found that determining pDNA concentrations using the Invitrogen Qubit HS assay was more reliable than using a Nanodrop spectrophotometer. Most likely, this is due to the tendency of traditional UV absorbance-based methods to overestimate sample concentrations due to the presence of contaminants. We found that there is an optimum in terms of amount of total pDNA and the DNA:PEI ratio at which maximum transfection efficiency is obtained, which correlated well with intracellular pDNA copy numbers and the % full particles. Moreover, an inverse relationship between total vg and % full particles was observed, meaning that parameters that increase total vg concomitantly decrease % full and vice versa. Independent of the serotype, the best results for rAAV production were obtained by decreasing the helper:production plasmid ratio, with a gradual decrease in total vg though concomitant increase in % full. In summary, we found that UV absorbance-based methods for pDNA quantification do not form a reliable basis for rAAV production process development, and that monitoring of intracellular pDNA copy numbers allow for better insight into the transfection and production process. Moreover, we observed an inverse relationship between total rAAV particle yield and percentage full. The driving force behind this relationship is yet unclear and the subject of follow-up research.

935 Omics Integration for Enhanced Peptide Discovery for Cancer Immunotherapy

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Cancer Immunotherapies have dramatically changed how cancer is treated nowadays. Advances in understanding anti-tumour immune response have provided novel strategies to precisely target malignant cells.In particular, the majority of anti-cancer vaccines and adoptive T-cell therapies rely on the accurate identification and validation of tumour-specific and tumour-associated antigens (TSAs and TAAs, respectively) displayed on the tumour surface through HLA-I molecules. Unfortunately, many antigens are poorly shared among patients and in addition, they show a significant degree of intra-tumoral heterogeneity. Furthermore, malignancy is associated with endogenous retroviruses (ERVs) reactivation upon genetic and epigenetic deregulation, a phenomenon observed in patients as well. Since ERVs and other classes of transposable elements (TEs) constitute over 45% of our genome while being unexpressed in normal cells, their reactivation could provide novel immunogenic HLA-I ligands, thus increasing the patient cohort'seligibility for therapeutic cancer vaccines.Hypomethylating agents (HMAs)(e.g. Azacytidine (AZA) and Decitabine (DAC)) are approved for the treatment of myelodysplastic syndrome (MS) and acute myeloid leukaemia (AML); the ability to further de-repress ERVs and activate anti-viral innate immunity in cancer cells has recently been speculated to be the actual mode of action of these drugs. Despite the promising results in preclinical studies, clinical knowledge regarding the HMA-induced set of ERV ligands

and their expression rate on the tumour surface is still limited. We here propose a deep Omic integration analysis approach for identification and selection to exploit HMAs' potential as a priming agent to unravel ERV targets for peptide-based immunotherapy.

936 Simple, Rapid and Robust Bioluminescent Cell-Based Assay for Detecting Neutralizing Antibodies Against AAV in Serum

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Adeno-associated viruses (AAV) are widely used vectors for gene therapy, capable of delivering transgenes as treatment for monogenic diseases. Individuals with pre-existing immunity to AAVs are less likely to benefit from AAV-based therapies due to both, a reduction in cellular AAV uptake and activation of a cytotoxic T-cell response to transduced cells, decreasing efficacy or changing the safety profile. A robust and reliable neutralizing antibody (NAb) assay is necessary to determine eligibility for treatment as well as to identify qualified individuals for clinical trials. Here we present a cell-based assay using NanoLuc® luciferase to detect NAbs in serum samples. This NanoLuc® AAV reporter system displays several advantages over existing assays such as: superior sensitivity, very low MOI required (100 - 3000 depending on serotypes), short 24h assay time, and detection using a standard luminometer. We tested 60 human serum samples for the presence of NAbs against a panel of AAV serotypes 1—10 and engineered variant AAV-DJ. We found that a large fraction of the population had pre-existing NAbs to at least one serotype, and of the pre-exposed individuals a majority displayed NAbs against multiple AAV serotypes. Interestingly, fewer samples were seropositive for AAV-DJ than for either parental serotype (AAV2, AAV8 or AAV9), highlighting the benefit of capsid engineering. Furthermore, a broad dynamic range of our assay enables categorizing the serum samples into 4 groups: negative, low, medium and high NAb tiers. Representative samples of each group were assayed for neutralizing titers (ND_{50}). The ND_{50} values of the samples showed agreement with their assigned groups. To demonstrate the robustness of our assay, we conducted a mouse study with 40 mice divided into 4 groups and administered AAV9 at varying doses. This study showed a strong correlation of NAb levels to the injected AAV viral loads. Taken together, our assay is rapid, highly sensitive and reliable; the NanoLuc® AAV reporter technology enables precise measurement of NAbs against AAVs in serum. Further validation of the assay is required for future use in a clinical setting.

937 New AAV Capsids with Increased Transduction Efficiencies of Human Airway Epithelia

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A major hurdle for cystic fibrosis (CF) gene therapy in the lung is delivery. Our goal is to achieve life-long correction from a single dose of aerosolized viral vector. As such, efficient delivery of a therapeutic gene or gene editing machinery to airway progenitor cells is critical. In this study, we use adeno-associated virus (AAV)-based vectors as vehicles for stable delivery of genes of interest to airway cells. Basal cells comprise an important progenitor population in the conducting airways and are responsible for replenishing multiple surface epithelial cell types. Thus, this cell population is an important gene therapy target. Here, we modify two capsids with demonstrated tropism for the conducting airways, AAV2.5T and AAV6.2, to further improve basal cell transduction. AAV2.5T and AAV6.2 are two capsid types that were generated by directed evolution to achieve improved airway tropism. AAV2.5T is a combination of AAV2 and 5 with a point mutation A581T. AAV6.2 is the AAV6 capsid with a mutated F129L residue. Both A581 and F129 are present in AAV6.2 and AAV2.5T. We first asked if incorporating reciprocal residues would increase airway tropism in primary surface epithelia and basal cells, termed AAV2.5T_F129L and AAV6.2_A581T. We next asked if modifying surface tyrosine residues would enhance transduction efficiencies as has been reported with the 6.2FF capsid (Y445F, Y731F). To this end, we generated a AAV2.5T_FF capsid by modifying Y437F and Y720F residues. Lastly, we generated combinatorial mutations, termed AAV2.5T_FFL and AAV6.2FFT. We compared transduction efficiencies on the apical surface of well differentiated primary airway epithelia as well as primary basal cells. GFP was quantified by flow cytometry 3 weeks post-transduction. Ratios of cell types were confirmed by flow cytometry. We observed increased transduction efficiencies using the AAV2.5T_F129L and AAV2.5T_FFL capsids compared to AAV2.5T alone. Additionally, we observed increased GFP expression with the AAV6.2FF capsid relative to AAV6.2. As expected, ratios of cell populations transduced remain unchanged with capsid modification. Apical transduction leads to mostly ciliated and secretory cells transduced, but basal cells are also transduced. Overall, AAV2.5T transduces surface epithelia with higher efficiencies than AAV6.2, however AAV6.2 better transduces airway basal cells. To assess AAV-mediated delivery in a CF model, we used a split intein system to deliver adenine base editors (ABEs) fused to a CRISPR/Cas9 nickase to primary CF cells. Using this approach, we observed efficient base editing at the R553X locus. Correcting both surface and airway progenitor cells are important for achieving life-long therapeutic benefits from cystic fibrosis gene therapy.

938 Increased Striatum Transduction with an Engineered AAV Capsid and Selective Transduction of Striatal Cholinergic Neurons Using a Truncated Human Choline Acetyltransferase Promoter

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Introduction. Adeno-associated virus (AAV) vectors are currently the most efficient option for intracranial gene therapies to treat

neurodegenerative disease. The striatum is the largest structure of the basal ganglia, and it plays a major role for movement and cognition. Alterations in the physiology of striatal cholinergic neurons have been found in human brains with motor diseases such as Parkinson's Disease. Increased efficacy and safety will depend upon robust and specific expression of therapeutic genes into specific cell-types within the human brain, including cholinergic neurons. In this study we set out with two objectives: (1) to identify capsids with broader transduction of the striatum upon intracranial injection in mice and (2) to test a truncated human choline acetyltransferase (ChAT) promoter which would allow efficient and selective transduction of cholinergic neurons. **Materials and Methods.** We first compared AAV9 and an engineered capsid, AAV-S, to mediate widespread reporter gene expression driven by the strong, broadly active CAG promoter (AAV-CAG-tdTomato) throughout the striatum in wild-type C57BL/6J mice. We also constructed an AAV expression plasmid containing a truncated human choline acetyltransferase (ChAT) promoter driving tdTomato expression (AAV-hChATp-tdTomato) to allow efficient and selective transduction of cholinergic neurons in mice. We used transgenic ChAT(BAC) eGFP mice with fluorescently labeled cholinergic neurons to allow accurate identification of transduced ChAT positive cells. We compared the ChAT promoter head-to-head with the CAG promoter to drive the expression of tdTomato after packaging into the tested AAV capsids. Mice were stereotactically injected with AAV-vectors into the striatum and 14-16 days post-injection, brain was collected and coronally sectioned. Confocal microscopy in Z-stack mode was utilized to quantify percentages of transduction efficiency and specificity. **Results.** We observed that AAV-S-CAG-tdTomato transduced a statistically significantly greater area, up to 3.2-fold, of the injected hemisphere primarily in the rostral direction compared to AAV9-CAG-tdTomato in C57BL/6 mice. The majority of transduced neurons appeared to be medium spiny neurons. For transduction of cholinergic neurons, we observed that AAV-hChATp-tdTomato mediated 7-fold higher selectivity of transduction of cholinergic neurons compared to AAV-CAG-tdTomato. Furthermore, the AAV9-hChAT-tdTomato vector increased the specificity and efficiency of transduction by 7-fold and 3-fold, respectively, compared to AAV9-CAG-tdTomato. **Discussion and conclusions.** The ability of AAV-S capsid to transduce a larger area of the murine striatum compared to AAV9, motivates further testing of AAV-S in large animals which may allow less invasive surgeries if less intracranial injections are required to transduce the striatum. The increased AAV transduction specificity and efficiency towards striatal cholinergic neurons *in-vivo* driven by the truncated ChAT demonstrates the utility of this AAV-vector as a tool for genetic modification of these cells and potential development of gene therapies targeted at cholinergic neurons.

939 Simultaneous Engagement of NK Cell Siglec-7 and FSHR Demonstrates a Promising Therapeutic Strategy Against Ovarian Cancer

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Targeted cancer immunotherapies have been a subject of significant clinical interest. Most of the recent developments have focused on unleashing tumor antigen-specific T-cell responses. Despite considerable success, their clinical benefit has been restricted to a subset of patients and selected tumor types, suggesting the need for additional complementary approaches. In this regard, NK and T cell strategies have unique cytokine profiles providing differential opportunities in specific therapeutic situations. Recently, NK cell engagers (NKCEs) have gained attention due to their anti-tumor activity and unique inflammatory profile. However, no NKCE has been clinically approved to date. NK cells, similar to T cells have described negative regulatory markers. One such potential marker is Siglec-7, which binds to sialoglycans on cancer cells. Attempts have been made to denude target cells of sialic acid moieties to prevent Siglec 7 from negatively regulating NK cell activation against tumors. We developed human Siglec-7 mAbs and show they can block negative signals driven by hypersyalylation of tumor cells. We hypothesized that Siglec-7 could be used as a NK cell engagement target as it would provide dual binding and activation. A unique clone DB7.2 exhibited high specificity for Siglec-7. We used this Fab and developed a NKCE by fusing with the Fab of a potent FSHR binding mAb (D2AP11) and studied this new NKCE for activity against diverse ovarian cancers (OCs). OC is highly heterogenous with poor survival post diagnosis using current modalities including traditional T cell-CPI blockade. We confirmed the NKCE would simultaneously bind to Siglec-7 and FSHR bringing NK cells into direct contact with FSHR+ OC cells. The NKCE was specific and potent in tumor cell killing, as evaluated by xCelligence RTCA against a panel of divergent OC lines carrying a wide array of important mutations (BRCA1&2, AKT, TP53, PIK3CA, BRAF). These tumors are patient derived and show resistance against different cancer drug targets (HSP90, HDAC, MTORC, DNA alkylating agents, EGFR, PARP, PI3K). The NKCE induced concentration dependent cell killing in OVISE-FSHR and OVCAR3 cells with EC50s of 142.87 pM and 236.6 pM, showing high potency of this new biologic. FSHR negative cells were not targeted by this NKCE, demonstrating that FSHR expression on target is required for NK mediated killing. In addition, the NKCE binding to tumor cells stimulated NK to produce sFas and granzyme A. It also attenuated tumor burden and significantly improved survival of mice in two different OC tumor challenge models. Additionally, the combination of FSHR targeted NK and T cell engagers resulted in synergistic killing of Kuramochi cells in presence of human PBMCs. This is the first demonstration of the potency of human anti-Siglec-7 for activation of NK cells to target pathogenic cells, and the first example of the development and study of bispecific Siglec-7 NK engagers. These results support Siglec7 NKCE targeting approaches as new tools for OC and potentially other cancers.

940 Scaling up Strategy for Oncolytic Herpes Simplex Virus Type-1 Vector Manufacturing

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Genetically modified recombinant herpes simplex virus type-1 (HSV-1) is widely used as an oncolytic virotherapy to stimulate anti-tumor immune responses by replicating in cancer cells. Conventional production of HSV-1 vectors uses adherent-based methods involving cell factories or roller bottles, which is time, people, space consuming and difficult for scaling up. In previous study, we reported the development of a high-titer, high quality production process of HSV-1 vectors utilizing Univercells Technologies' scale-X™ hydro system. In this study, we demonstrated the scalability of the previously developed HSV-1 production process on scale-X™ carbo system with consistent quality profiles. The process on adherent Vero cells culturing in fixed bed bioreactors was scaled-up to 30 sqm total growth surface area, which is equivalent to 350 roller bottles or twelve 40-layers large cell factories. The peak cell density and peak virus titer were measured and critical quality attributes including residual host cell DNA and host cell protein were determined. The results indicated that the virus yield from a 30 sqm fixed bed culture is comparable to traditional roller bottle, which significantly reducing the requirements on manpower, material, and space. The yield and quality of vector production showed consistency when scaling up from hydro bioreactor to scale-X™ carbo system, and the consistency is likely to be there by further scaling up to 600 sqm scale-X™ system.

941 DMSO Promotes AAV Production in Scalable Suspended HEK293 Cells

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AAV production for gene therapies faces the challenge of high-cost manufacturing and improving the yields can potentially lower the cost. We screened for molecules that could increase AAV production in scalable HEK293 suspension cultures. Among these molecules, DMSO was identified to robustly increase AAV production regardless of transfection reagents or AAV serotypes were utilized. DMSO has been reported to boost transfection efficiency in multiple adherent cell lines by high-concentration DMSO shock. This type of DMSO shock is not applicable to suspension cultures due to the difficulty of completing the process within a minute, and that changing the medium would require centrifugation and re-suspension, which would distress the cells. We treated the cells with different concentrations of DMSO for 72 hours and measured their viability. We found that HEK293 suspension cells can well-tolerate 2% DMSO. Therefore, various DMSO concentrations that were ≤2% were used to treat the cells during AAV packaging. There was a dose-dependent increase in AAV vector genome (vg) titer. This increase was stably observed with different transfection reagents, different AAV serotypes, and different scales. An additional benefit is that DMSO does not affect downstream purification and can be easily removed from the product. In addition to increasing in vg titer, there was a robust increase of full capsid ratio, which is an important parameter for AAV purification. In summary, DMSO can boost AAV production and potentially reduce manufacturing cost.

942 A Low-Cost, Highly Stable, and Scalable Assay to Interrogate Effectors of rAAV Transduction

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Recombinant adeno-associated viral (rAAV) vectors are increasingly popular platforms for *in-vivo* gene therapy. Novel rAAV capsids and transgene components that increase transduction efficiency and cell type specificity are highly active areas of research that can benefit from effective high-throughput transduction assays to screen large arrays of vectors. Pharmacological agents can also increase transgene expression but have been less explored due to the lack of a robust platform for small molecule screens. Here, we describe a method to evaluate rAAV transduction using a homemade reporter assay we call *LacZ*-Activated Fluorescence Assay (LAFA). Briefly, cells are transduced with *lacZ*-packaged rAAV, lysed, and mixed with the substrate 4-methylumbelliferyl-ß-D-galactopyranoside (MUG), a fluorogenic galactose analog. Upon cleavage by -galactosidase, 4-methylumbelliferone (MU) is released and fluoresces blue. The cost-effectiveness, ease of use, and practicality of LAFA pose several advantages over ubiquitously popular luciferase-based reporter assays. Our homemade assay is roughly 50-times less expensive than commercial luciferase or fluorescence kits, requires minimal reagent preparation, and can be scaled up to a 384-well plate format. Because the fluorescent readout is stable for hours compared to minutes in luciferase assays, LAFA does not require the use of plate readers with reagent injectors and is thus better suited to scale-up and use in liquid handling robotic platforms. Additionally, MUG may be replaced by fluorogenic galactose analogs with green, red, or far-red spectra, owing to the versatility of the approach and providing researchers the ability to customize the assay based on their unique needs. Using LAFA, we assayed transduction by a trans-splicing dual vector, an approach previously engineered by others that can double the transgene size deliverable by rAAV. The 5' half of *lacZ* is flanked by a promoter and splice donor, packaged in an AAV2 capsid, and co-delivered with a separate AAV2 vector packaging the 3' half of *lacZ* flanked by a splice acceptor and polyA. As expected, transduction of cultured cells by the dual vector was 10-fold lower than by an unsplit, single *lacZ* packaged vector. We then used LAFA as a readout to test the effects on transduction of small molecular inhibitors targeting DNA damage repair (DDR) factors hypothesized to be involved in vector genome processing. Inhibiting the non-homologous end joining (NHEJ) protein DNA-PK results in a 10-fold reduction in dual vector transduction. Conversely, by inhibiting the homology-directed repair (HDR) protein Rad51, we observe a 10-fold increase in dual vector transduction, with RFUs comparable to unsplit *lacZ* single vectors. All observations were dose-dependent with respect to both the small molecule and vector. These results suggest that NHEJ supports dual vector transduction, while HDR impedes it. The versatility and accessibility of LAFA enables

studies that will advance our knowledge of rAAV biology and progress the development of novel vector designs and transduction-enhancing drug discovery via high-throughput screens.

943 Assessing the Effect of Dosage and Injection Route on AAV Gene Therapy in the Treatment of Feline GM2 Gangliosidosis

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The GM2 gangliosidoses (GM2) are a class of fatal neurodegenerative disorders resulting from a mutation in the gene encoding β-N-acetylhexosaminidase (Hex). The naturally occurring feline model of GM2 is an effective animal model for characterizing disease progression and evaluating treatment efficacy. Adeno-associated viral (AAV) gene therapy for GM2 aims to restore Hex and minimize disease progression. Injection route is an important consideration in AAV therapy because the vector must reach areas of the body most relevant to the disease pathology. Moreover, AAV dosage must be high enough to generate a therapeutic effect without causing adverse effects. Intracranial injection of AAV into the thalamus and deep cerebellar nuclei is effective in increasing in the lifespan of GM2 cats, but the procedure is invasive and requires a craniotomy for delivery to the brain parenchyma. Injection of AAV into the cerebrospinal fluid via the cisterna magna (CM) is less invasive and may improve biodistribution to certain parts of the central nervous system. Intravenous injection is an even less invasive injection route and could be especially effective in treating peripheral organs. Furthermore, delivering vector via both CM and IV could yield the positive effects of both injection routes at the same time. This study compares the effects of AAV dosage and injection route on survival and disease progression in GM2 cats. Each cohort received a low dose (LD) of 5×10^{13} vector genomes/kg body weight (vg/kg) or a high dose (HD) of 2×10^{14} vg/kg, via CM, IV, or combined (CM+IV) routes. For the CM+IV injections, half of the total dose was administered via each injection route. Cats were treated at 1.15 ± 0.2 months of age. Animals in each cohort were observed either for 16 weeks post-treatment or until they reached humane endpoint. 7T MRI was used to assess neurodegeneration across cohorts. Magnetic resonance spectroscopy (MRS) was used to quantify several metabolites that are biomarkers for brain health. MRI showed delayed neurodegeneration in all cohorts, to some degree. When compared to normal cats at 4 months, the typical GM2 humane endpoint, untreated GM2 cats have isointense (equal intensity) gray and white matter. All treatment cohorts showed preservation of white matter at approximately 4 months. However, neurodegeneration and atrophy had progressed substantially in all treatment cohorts once the humane endpoint was reached (see survival below). At 4 months, MRI and MRS data for the treated cohorts correlated with delayed clinical symptoms, such as tremors and inability to stand. Ultimately, untreated GM2 cats survived 4.4±0.6 months (n=9), CM LD cats (n=4) survived 9.4±4.2 months, CM+IV LD cats (n=3) survived 14.1±4.4 months, CM+IV HD cats (n=3) currently have survived 13.1±3.3 months (study ongoing), IV LD cats (n=4) survived 8.3±1.2 months, and IV HD cats (n=4) survived 12.4±2.8 months. This study indicates that all dosages and treatment routes significantly increase the lifespan of GM2 cats and delay neurological disease progression when compared to untreated cats. This study indicates that all dosages and treatment routes significantly increase the lifespan of GM2 cats and delay neurological disease progression when compared to untreated cats.

944 Effect of an Anti-VSV-GP Antibody on Viral Pharmacokinectics and Biodistribution

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Introduction: VSV-GP is a vesicular stomatitis virus (VSV) which has been pseudotyped with the glycoprotein (GP) from lymphocytic choriomeningitis virus (LCMV). VSV-GP is a novel oncolytic virus with potential therapeutic application. Regarding oncolytic virus based therapies, anti-viral antibodies may impact pharmacokinetics (PK) and biodistribution (BD) of these drugs. Anti-viral antibodies can also impact the safety and efficacy of oncolytic viruses. Therefore, it needs to be determined if these antibodies will be generated and if so what is the potential impact. The objective of these studies was to investigate the development of anti-viral antibodies to VSV-GP in mice and the effect of said antibodies on VSV-GP PK and BD. **Methods:** Female BALB/c mice were intravenously administered multiple doses of 10^8 TCID50 VSV-GP over a 2 week period. After the mice were rested for 2 weeks, they were sacrificed. Their serum was collected and analyzed via an MSD based indirect ELISA utilizing VSV N protein as coating antigen. In a separate study, an anti-VSV N IgG2a mouse monoclonal antibody (Clone 10G4, Kerafast, Cat # EB0009) was pre-incubated with VSV-GP and then administered in female BALB/c mice. Mice treated with VSV-GP alone were used as a control. After 1, 24, and 72 hrs, the mice were sacrificed and their blood, spleen, and liver tissue analyzed via RT-qPCR to assess viral titers. **Results:** All mice treated with VSV-GP developed anti-viral antibodies. Specifically, an antibody response against the nucleoprotein was detected. Coadministration of anti-VSV N antibody with VSV-GP in mice, produced similar viral genome copies as virus only treated mice in both spleen and liver tissue. However, in anti-N antibody treated mice, there was a distinct and log fold reduction in viral genome copies detected in the blood at 24hrs post infection compared to virus only treated mice. **Conclusions:** VSV-GP administration leads to anti-viral antibody development to VSV N protein. While all mice did develop a detectable antibody response to the N protein, the signal was low in this assay developed to evaluate anti-VSV-GP antibody responses. Anti-N antibody had no effect on biodistribution of VSV-GP in spleen and liver tissue. VSV-GP blood PK was affected by anti-VSV N antibody treatment in mice. Since there was no difference in genome copies in the blood at 1hr post infection, anti-N antibody was not disrupting viral egress from the blood. We postulate that administration of anti-N antibody leads to increased clearance of viral genomes that are associated with N protein, which has been released from infected cells.

945 Development of a Novel Non-Viral Gene Therapy Platform

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Gene therapy is a promising approach for treatment of severe bleeding disorders. AAV-based gene therapies have shown some success in this regard, though age restrictions, pre-existing capsid immunity, limited durability, and an inability to re-dose represent challenges to this platform. The super piggyBac® DNA insertion system is a transposon-based gene therapy platform that enables stable integration of the therapeutic transgene into the genome, thereby offering the potential for durable and lifelong activity. This platform requires two components: the super piggyBac transposase (SPB) and a piggyBac transposon comprising the therapeutic transgene expression cassette. The transposase functions by excising DNA cargo and inserting it into the genome, and can be efficiently delivered as mRNA. One key advantage is the super piggyBac platform accommodates large cargo; this is especially advantageous for diseases such as hemophilia A in which the transgene (hFVIII) is especially large. We hypothesized that combining the super piggyBac DNA insertion system with a non-viral delivery system could enable us to deploy this platform unconstrained by the cargo capacity limitations of AAV-based delivery systems, and potentially enable repeat dosing. The lipid nanoparticle (LNP) is a mature non-viral delivery platform that has been clinically successful for liver-specific delivery of siRNA and mRNA. The implementation of the LNP platform to deliver DNA, however, has been challenging. Successful implementation of our super piggyBac system requires: 1) the development of LNPs capable of effectively and safely delivering the transposase mRNA and donor DNA template to the same target cells, 2) optimization of the transposon DNA format and sequence, and 3) optimization of the SPB mRNA to maximize transposase expression to give optimal excision and insertion of our gene of interest. Using LNPs optimized using Design of Experiments (DOE), we explored (1) dual formulation, in which the transposon DNA and transposase mRNA are encapsulated in separate LNPs, and (2) co-formulation, in which the DNA and mRNA are encapsulated together within the same LNP. We observed substantial hFVIII expression (>100% of normal) in juvenile wild-type mice following a single intravenous dose. Optimization of the transposon DNA: transposase mRNA ratio resulted in a further 8-fold increase in FVIII expression for both formulation strategies. Delivery of the hFVIII DNA alone or in concert with a control transposase (unable to perform genomic integration) resulted in significantly (>10X) lower hFVIII expression than using functional SPB. Vector copy numbers remained low, with an insertion profile consistent with previous studies. Production of hFVIII in the expected therapeutic range (10-50%) was also confirmed in a mouse model of severe hemophilia A. Contrary to conventional AAV-based gene therapies, this fully non-viral approach also supports repeat dosing. Repeated administration of the transposon/transposase nanoparticle system over 10 days resulted in dose-proportional increases in hFVIII antigen levels. In conclusion, we have demonstrated the feasibility of a non-viral approach using novel LNPs for treating severe bleeding disorders using the super pigyBac DNA insertion system, with the potential for repeat dosing.

946 Preclinical Development of an Adeno Associated Vector-Based Gene Therapy (SENS-501) for the Autosomal Recessive Non-Syndromic Deafness 9 (DFNB9)

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Background: Autosomal recessive genetic forms (DFNB) account for most cases of profound congenital deafness. We focus here on the *Otoferlin* gene underlying DFNB9, one of the most frequent genetic forms of congenital deafness. Otoferlin is a calcium sensor protein critical for the transmission of the signal from inner hair cells (IHC) to the spiral ganglion neurons. DFNB9 is caused by pathogenic biallelic loss of function variations in *OTOF* gene leading to the failure of synaptic transmission, resulting in congenital severe-to-profound auditory neuropathy. Cochlear implantation is the only option proposed to young patients thus far. Although this medical device improves the quality of life and language acquisition, hearing quality is limited, and a treatment for DFNB9 is necessary to address this unmet medical need. **Method:** We have developed SENS-501 as a dual AAV (Adeno Associated Virus) approach using two different recombinant vectors, one containing the 5' and the other the 3' portions of *Otoferlin* cDNA. SENS-501 was delivered into congenitally deaf DFNB9 mutant mouse inner ears through the round window at different doses. The therapeutic candidate, and a FLAG-tagged surrogate, was validated through demonstration of *Otoferlin* expression and integrity upon reconstitution of the full-length sequence in vitro and in vivo both in mice and NHP using immunohistochemistry. Dose-response experiments, early biodistribution studies after intracochlear injection were performed in mice and non-human primates (NHP). In primates, the surgical method and the delivery device were the ones envisioned in human. Biodistribution studies were conducted by qPCR and RT-qPCR. The reversal of the deafness phenotype in *Otof*$^{-/-}$ mutant mice was evaluated through the assessment of auditory brainstem response (ABR) recordings after intra-cochlear administration either pre-hearing onset, or post-hearing onset and acoustic startle test, a behavioral test allowing to demonstrate efficient auditory processing. **Results:** IHC-restricted *Otoferlin* expression and good preliminary tolerability were demonstrated in both mice and NHP. Post-natal intracochlear injection of SENS-501 into the DFNB9 mutant mouse

inner ear led to improvement of hearing thresholds and behavioral response as early as 3 weeks post-injection. Long-term ABR recovery was achieved in a dose dependent manner, with efficacy demonstrated for at least ten months. Dose-range, early biodistribution, as well as preliminary safety studies including immunogenicity with SENS-501 helped to design the ongoing GLP toxicity and biodistribution studies. **Conclusions:** SENS-501 appears safe and well tolerated. The selected AAV vector components allow to efficiently target IHC at levels compatible with therapeutic intervention in human and provide long-term efficacy data in DFNB9 mutant mouse model, which constitute a major step toward our future clinical trials to restore physiological hearing in DFNB9 patients.

947 Stable Producer Cell Lines for Production of Lentiviral Vectors for CAR T Cell Therapies

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The market approval of chimeric antigen receptor (CAR) T cell therapies together with the continuous emerging number of CAR-based ex-vivo and in-vivo targeted products in development has created increased demand for the supply of large quantities of high-quality grade lentiviral vectors (LVV). Production of LVV using producer cell lines (PCLs) is desirable due to reduced costs, improved batch consistency and further up-scaled and streamlined production processes when compared to LVV produced by the standard transient transfection manufacturing process. However, the development of PCL LV-CAR cells lines has been extremely challenging as it was found that constitutive expression of the CAR molecules often elicits a detrimental effect on the survival of PCLs capable of producing high titre LVV. The combination of an improved version of the TRiP system™ able to efficiently repress CAR expression in a stable cell line, with a well-established cell line development platform, have enabled the successful generation of high titre suspension PCL LV-CAR clones with accelerated timelines. To complement this, a new manufacturing process has been developed, which takes advantage of advances in perfusion technology to support LVV production at higher cell densities than those associated with traditional batch and fed batch production approaches. This new process has been scaled to 5L bioreactors using PCLs, delivering a significant improvement in LVV-yields compared to batch processes and with equivalent downstream recoveries. As such, this new process represents the next generation of LVV manufacturing processes, yielding high LV-CAR titres from inducible PCLs at large-scale. It is anticipated that this PCL LVV production platform will support increased yields at reduced cost enabling more patients to be treated with these life changing cell and gene therapies.

948 Profiling the Tropism of AAV Variants for Various Mouse Tissues Using Directed Evolution

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Recently, adeno-associated virus (AAV)-mediated gene therapy approaches have emerged as a promising and effective means for treating a variety of incurable human diseases due to their safety and delivery efficiency. However, naturally occurring AAVs have a problem with off-targeting due to broad tissue specificity, which limits administration routes and reduces stability. Directed evolution can efficiently identify more specific AAV variants for particular tissues, addressing the off-targeting problem of wild-type AAVs. We attached a 25 bp nucleotide barcode sequence to a library of AAV capsid variants, created through error-prone PCR, to determine their specificity for various tissues. The barcode was uniquely matched to each capsid molecule in the library and used for nanopore sequencing, a next-generation sequencing method, after *in vivo* selection during the directed evolution process in mice. These results allowed us to establish an AAV capsid gene mutation profile for each mouse tissue.

949 Structure Function Correlates of New AAV Variants Evolved in Human Brain Explants

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Adeno-Associated Virus (AAV) is a commensal virus in mammals that has shown significant promise as a vector for human gene therapy. A major challenge facing effective clinical translation is the species-specific variations in tropism and efficiency across both natural and novel AAVs. The AAV capsid and genome regulatory elements are critical determinants of vector performance. In particular, data in human tissue are scarce, and our understanding of transduction in human CNS cell types is still developing. Here we take steps to unravel AAV capsid biology in human brain tissue by cycling multiple AAV libraries based on serotypes 6,8, and 9. Briefly, enabled by an IRB approved protocol following patient surgery for intractable epilepsy, we subjected patient-donated, resected cortical tissue to AAV library cycling. Newly evolved AAV variants obtained from the human cortex demonstrated markedly improved transduction in brain slice cultures. We observe serotype and variant capsid-dependent patterns of widespread, pan-neuronal to glial transgene expression. Notably, we identify different capsid surface domains contributing to improved transduction efficiencies in primary human neurons and ability to penetrate deeply within cortical slices. We also confirm robust gene transfer of several human cortical tissue-derived AAVs compared to natural serotypes in mice following intracerebroventricular injection. Further, using transcriptomic analysis of barcoded AAV capsids in human cortical slices and the mouse brain, we bridge structural

determinants with observed cellular tropisms. Understanding AAV vector biology in human tissues alongside other preclinical animal models could improve predictive modeling and clinical translation of CNS gene therapies.

950 A Novel Feeder-Free Platform for Immune Cell Manufacturing

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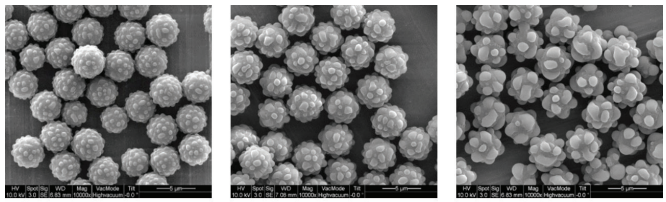
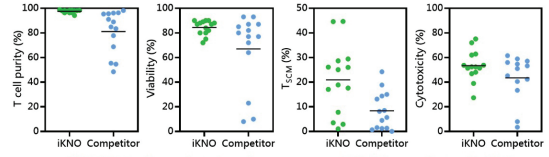
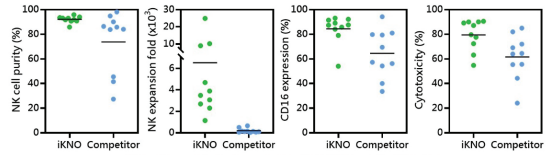
In recent years, cellular immunotherapies have revolutionized the strategy for cancer treatment. Antibody-coated magnetic beads have become the most widely used artificial antigen-presenting cells (APCs) for CAR-T cell manufacturing, and all the commercially available products are spherical in shape. Inspired by the natural morphology of dendritic cells, micrometer-sized magnetic beads with tailorable sizes and shapes were fabricated for the first time, named iKNOBeads. The bumpy body creates a larger contact area for cell interactions, which improves activation efficiency and reduces the amount of beads used. The reactive functional groups grafted on the polymeric surface make it easy to modify various stimulatory ligands. The uniformity of bead size provides batch-to-batch reproducibility. The magnetic property of beads allows for ease of separation. Based on the advantages of iKNOBeads, we have successfully developed a 7-day process for CAR-T cell manufacturing. In a DLBCL-patient study, CAR-T cells exhibit higher purity, quality, and in-vitro cytotoxicity than the product manufactured by a 10-day process using commercial magnetic beads. Natural killer (NK) cells are emerging as a candidate for next-generation allogeneic cell immunotherapy. Genetically engineered feeder cells enabling an effective expansion of NK cells have become the market's mainstream. However, there are safety concerns regarding contaminating cells in the final therapeutic product. We have screened numerous stimulatory candidates to be modified and proved that iKNOBeads has enormous potential for NK cell manufacturing. In a healthy-donor and breast cancer-patient study, the expansion capability, purity, and cytotoxicity of NK cells expanded with iKNOBeads are superior to that of commercially available feeder-free products and are comparable to that of feeder cells reported. These results show that iKNOBeads offers a more effective and economic feeder-free platform for generating high quality and quantity immune cells for therapy. GMP-compliant production of iKNOBeads has been established for clinical applications.



Electronic microscopy images of the core of iKNOBeads



CD19 CAR-T cell manufacturing using cryopreserved PBMC from patients with DLBCL
iKNOBeads: bead/cell=1:1, 7-day culture; Competitor: bead/cell=3:1, 10-day culture; Target cells: NALM-6, E:T=1:1, 24 h



NK cell manufacturing using fresh PBMC from healthy donors
14-day culture, iKNOBeads: bead/cell=1:1; Competitor: bead/cell=0.5:1; Target cells: MDA-MB-231, E:T=1:1, 4 h

951 Single-Cut Gene Editing Therapy for Duchenne Muscular Dystrophy via a Single AAV Vector

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Background: Duchenne muscular dystrophy (DMD) is an X-linked fatal disease arising from genetic mutations in the *DMD* gene and has become the most common neuromuscular disorder in childhood whose global prevalence reaches 1 in ~3500 male births. However, there is no curative therapy for this devastating disease at present. Among thousands of DMD-causing mutations, the skipping and reframing of exon 51 are estimated to therapeutically benefit at least 14% of all DMD cases worldwide. Thus far, an antisense oligonucleotide medicine (also known as Eteplirsen) has been approved to treat DMD patients with particular mutations in the exon 45-55 hotspot region by promoting dystrophin production and restoring the translational opening reading frame (ORF) of DMD through exon 51 skipping at the transcription level. However, Eteplirsen-treated patients had mean increase in dystrophin protein expression at 0.22 - 0.32% of the normal levels after 48 weeks of weekly intravenous infusion. The wide application of Eteplirsen is greatly limited by many factors such as high cost, short half-life, and poor delivery efficiency to the heart. Therefore, there is a huge unmet medical need to explore innovative yet efficient therapeutic approaches to correct genetic mutations and restore dystrophin expression and muscle functions in individuals with DMD. **Methods**: Humanized DMD mice were generated using targeted deletion of mouse exon51 and exon52 via replacement with human exon51, which disrupted the dystrophin ORFs. We explored the efficiency of proprietary hfCas12Max-mediated single-cut genome editing in human *DMD* exon51 splice donor (SD) site *in vitro,* and an all-in-one AAV vector system was chosen for in vivo delivery of hfCas12Max and a gRNA targeting the SD site of human exon51 to manifest the *in vivo* single-cut efficiency. **Results:** The genetically humanized DMD mice exhibited highly similar phenotypes with that of humans and were suitable for subsequent analysis of potential interventions strategies. There was a high efficiency of hfCas12Max-

mediated single-cut genome editing in human *DMD* exon51 SD site *in vitro*. Systemic *in-vivo* hfCas12Max gene editing efficiently restored dystrophin expression and ameliorated pathologic hallmarks of DMD, including histopathology and grip strength in this mouse model (Fig. 1). **Conclusions**: This unique DMD mouse model with the human genomic sequence allows *in vivo* assessment of clinically relevant gene editing strategies as well as other therapeutic approaches. Research on improving present antisense-based therapeutic strategies represents a significant step toward therapeutic translation of gene editing correction of patients with DMD.

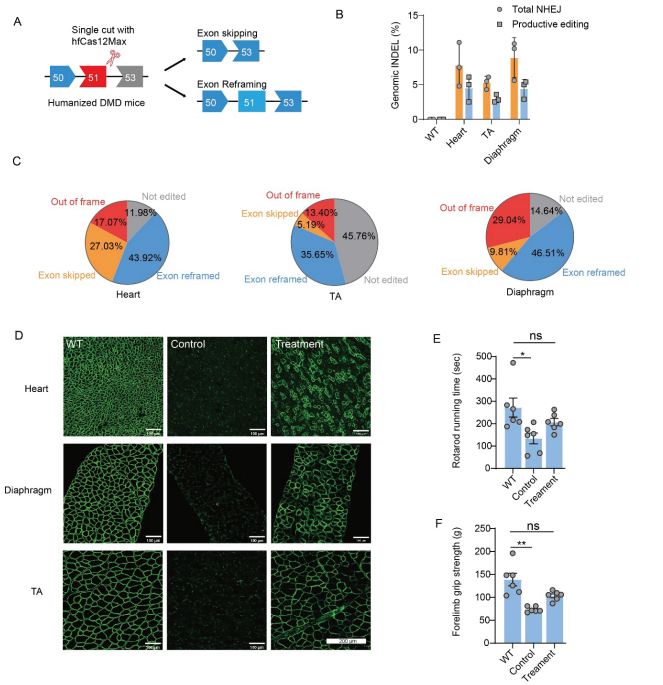

Figure 1. Efficient *in vivo* restoration of dystrophin expression and muscle functions by hfCas12Max in the humanized DMD mouse model. A, hfCas12Max-mediated single-cut gene editing strategy was designed to restore the open reading frame of the dystrophin gene by exon skipping and exon reframing. B, The percentages of genomic editing indels in the heart, TA and DI from DMD mice treated with AAV or saline. C, Deep sequencing analysis of RNA from the heart, TA, and DI of AAV- or saline-treated humanized DMD mice. D, Immunohistochemistry analysis of dystrophin expression in the heart, DI, and TA of WT, DMD mice infused with AAV or saline. Dystrophin was shown in green. Scale bars, 200 μm. Rotarod (E) and forelimb grip strength (F) were measured in WT, DMD mice with AAV or saline treatment. Data shown as mean ± SEM. Significance was indicated by the asterisk. Different asterisk represented the statistical significance among multiple groups by ANOVA.

952 An Open-Label, Dose-Escalation, Phase 1a Study to Evaluate Safety, Tolerability, and Exploratory Efficacy of Intravenous Injection of Allogenic Early-Passage Mesenchymal Stem Cells (MSCs), Derived from Wharton's Jelly in the Umbilical Cord, in Patients with Duchenne Muscular Dystrophy

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Introduction Duchenne Muscular Dystrophy (DMD) is a genetic disease with progressive muscle degeneration caused by mutations in the dystrophin gene. As an X-linked recessive disease, it affects approximately 1 out of every 5,000 male infants and about 20,000 newborns worldwide each year. The study was an open-label, dose-escalation, phase 1a clinical trial in DMD patients with EN001, a next-generation stem cell therapy. EN001 is an allogeneic early-passage MSC derived from Wharton's Jelly in the umbilical cord which demonstrated a high treatment efficacy for DMD in preclinical studies. Objective To evaluate the safety, tolerability, and exploratory efficacy of EN001. Methods A total of 6 male participants aged between 2 to 18 years who were genetically confirmed as DMD enrolled in a 12-week study and a long-term (5 years) follow-up study. Three participants received EN001 with a low-dose (5.0x105 cells/kg) once and the other 3 participants with a high-dose (2.5x106 cells/kg) once intravenously on Day 1.Adverse events with a grade 3 or higher in national Cancer Institute Common Terminology Criteria for Adverse Event (NCI-CTCAE) were defined as a dose-limiting toxicity (DLT) during the first 2 weeks after the EN001 administration. Serum creatine kinase (CK) level, North Star Ambulatory Assessment (NSAA), Six Minute Walk Test (6MWT), muscle strength, and pulmonary function were measured within the 12 weeks study period to evaluate the exploratory efficacy. Results DLTs nor serious adverse events (SAE) were not observed in a 12-week study. And Infusion-related reactions were not observed. Six treatment-emergent adverse (TEAE) events were reported as mild and the patients recovered within 5 days from the onset.Interestingly, decrease in the level of serum CK was observed during the study period in all patients. In NSAA, one patient in the low-dose group showed an improvement from 1 (Modified method but achieves goal with no physical assistance) to 2 ('Normal' - no obvious modification of activity) in item 11 "Gets to sitting", and a patient in the high-dose group revealed improvement from 0 (Unable to achieve goal independently) to 1 (Modified method but achieves goal with no physical assistance) in both item 6 "Climb box step - right" and item 9 "Descend box step - Left". The overall score of all patients was not changed compared to the baseline, which may indicate that the progression of the disease was delayed. In addition, two patients in the low-dose group and one patient in the high-dose group showed improvement in forced vital capacity in the Spirometry. The muscle strength of the left knee extensors was improved in one patient in the low-dose group and two patients in the high-dose group, and that of the right knee extensors was improved in all 6 patients. Conclusions EN001 was safe and well-tolerated at both low-dose (5.0x105 cells/kg) and high-dose (2.5x106 cells/kg) for DMD patients. In addition, the data on treatment efficacy indicates that EN001 improved ambulatory capacity, pulmonary function, and muscle strength in some patients.This study suggests that allogeneic early-passage MSCs derived from Wharton's Jelly might have a potent therapeutic effect for DMD treatment and can be used in all subtype-specific DMD patients.

953 A Streamlined AAV Purification Process by Direct Loading of Filtrated Harvest Materials onto an Affinity Chromatography Column

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GMP, clinical grade, adeno-associated virus (AAV) vectors used in various modalities such as gene therapies and vaccines, need to meet stringent quality and purity standards for clinical use. Generally, the downstream processing of AAV products involves cell lysis, depth filtration, and ultracentrifugation/diafiltration (UFDF) before loading onto an affinity chromatography column. The UFDF unit operation often utilizes expensive membrane cartridges, complex equipment, and results in long processing time due to slow filtration speed and

large harvest volume. We developed a simplified AAV downstream processing and purification platform which does not include the UFDF operation. The clarified AAV harvest is loaded directly onto an AAVx or AVB column. This process platform has been tested on various AAV serotypes such as AAV 2, 5, 6, 8, 9 and DJ. The purification step yield and removal of impurities, such as host cell proteins, nucleases, and residual DNA for the simplified purification process were evaluated. Using an enhanced column cleaning procedure, the purification efficiency and binding capacity of AAVx affinity resin were not affected by this simplified, direct loading method. As the result, our improved downstream AAV purification platform is shown to not only achieve a similar or higher product yield, but also use less time, with lower cost and labor compared to a general process with a UFDF step.

954 Modeling Antigen-Specific CAR T-Cell Inhibitors in Nonhuman Primates

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Introduction: Translating successful chimeric antigen receptor (CAR) T-cell therapies from B cell malignancies to solid tumors and infectious diseases has proven challenging. To identify factors associated with successful and suboptimal strategies *in vivo*, we have developed respective nonhuman primate (NHP) models of CD20$^+$ B-cell-specific and HIV-1-specific CAR T-cell therapies. Our HIV model is designed to overcome inefficient CAR-mediated clearance of latently infected cells in secondary lymphoid tissues by boosting CAR T-cells with cell-associated viral Envelope (Env) protein *in vivo*. Here, we used NHP B cell-specific CAR T-cells as a benchmark to identify barriers associated with HIV-specific CAR T-cell trafficking to tissues, recognition of target antigen, and impacts of cell-free vs. cell-associated antigen. **Methods:** A total of 23 rhesus macaques were infected with one of 2 HIV-like viruses to model lower and higher levels of viral antigenemia, then suppressed by antiretroviral therapy (ART) for at least 7 months prior to HIV-specific CAR T-cell dosing. CAR infusion was followed by infusion of irradiated antigen-expressing cells (K562-Env), and ART cessation leading to rebound viremia. To support CAR T cell persistence in low-antigen environments, we also evaluated i) co-infusion of T cells expressing 4-1BB- and CD28-based CARs, and ii) arrayed CRISPR-edited CAR T-cells designed to overcome immune exhaustion. CAR expression and trafficking was quantified via direct immunolabeling of the CAR molecule, ELISA assays quantified humoral responses to antigen boosting, and next generation sequencing was used to measure persistence of immune checkpoint-edited cells. **Results:** Whereas CD20 CAR T cells consistently expanded and depleted CD20$^+$ B cell targets in NHP, virus-specific CAR T-cell function varied substantially between animals. Manufacturing data and functional assays validated the quality and potency of our respective NHP CAR T-cell infusion products. Cell-associated Env boosting triggered a significant increase in serum anti-Env antibody titers, consistent with *ex vivo* data showing preferential impacts of cell-associated antigen on virus-specific CAR T-cell function. Degree of CAR expansion in peripheral blood was the strongest predictor of target depletion efficiency in our model, which was not impacted by CAR domain structure or CRISPR editing. Intriguingly, we observe only minimal trafficking of CAR$^+$ cells to secondary lymphoid tissues, independent of target depletion efficiency. **Conclusions:** Our unique and highly informative large animal models draw sharp contrasts between the *in vivo* function of CAR T-cells in antigen-replete (CD20$^+$ B-cells) vs. antigen-sparse settings (ART-suppressed viral infection). In addition to a paucity of antigen, our evidence suggests that virus-specific CARs must overcome inhibitory effects of cell-free antigen (namely infectious virions) and virus-specific antibodies, which could both interfere with CAR T-cell recognition of cell-associated antigen. Immunocompetent preclinical models such as ours will be essential in order to characterize and overcome suppressive immune microenvironments that may downregulate CAR T-cell function in numerous clinical settings.

955 Classification Modeling to Identify Patients at Risk for Neurotoxicity and CRS Following Axicabtagene Ciloleucel (axi-cel) Treatment in 2nd Line R/R LBCL

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Background: Axi-cel is an autologous antiCD19 chimeric antigen receptor T-cell therapy approved for the treatment of relapsed or refractory large B-cell lymphoma after ≥1 lines of systemic therapy. ZUMA-7, a global Phase 3 randomized study, showed superiority of axi-cel over standard second-line therapy (N=359; event-free survival [EFS] HR 0.398, P<0.0001, Locke et al. *NEJM*. 2021). Post-axi-cel cytokine release syndrome (CRS) and neurologic events (NE) were reported in 92% and 61% of patients, and rates of high-grade (Grade 3+) events were 6% and 21%, respectively. While the pathophysiology of CRS is well documented, the mechanisms underlying NE remain elusive. Hence we investigated biomarkers and classification models to identify *a priori* those patients more likely to experience high-grade post-axi-cel NE toxicity - as well as patients who may experience low/no post-axi-cel toxicity and may be amenable to treatment in the outpatient setting.**Methods:** For classification models, we considered biomarkers collected at baseline (i.e., prior to lymphodepletion) or on Day 0 (prior to axi-cel), which included demographics, clinical and disease characteristics, routine blood chemistry and hematology, cytokines and other pharmacodynamic (PD) markers, and product attributes. To account for the impact of lymphodepletion, we further examined fold changes of routine lab and PD markers between baseline and Day 0. Multiple machine learning methods (e.g., conditional random forest, XG Boost) were used to select features of interest for classifying patients by outcomes, where a data-driven approach was implemented (i.e., a set of presumed clinically-relevant biomarkers was not selected *a priori*). A logistic classifier was trained and performance measures were evaluated in the hold-out test set (70%-30% split).**Results:** Thirty-six of the 170 axi-cel-treated patients (21%) experienced Grade 3+ NE post-axi-cel. Sixty-seven (39%) axi-cel-treated patients had low toxicity defined as a maximum of Grade 1 CRS and no NE within 7 days post-axi-cel. We determined that top-performing models for

high-grade NE (Grade 3+) include between 3 and 5 biomarkers (e.g., standard clinical labs and disease characteristics) and have a high negative predictive value (NPV; mean NPV in test set: 0.94; 90% empirical CI: 0.90-1.0), while also achieving high sensitivity (mean in test set: 0.88; 90% empirical CI: 0.80-1.0). Best-performing classifiers for low-grade toxicity included between 3 and 6 biomarkers (e.g., PD markers and product features) and had modest positive predictive value (PPV: mean PPV in test set: 0.69; 90% empirical CI: 0.54-0.87), and achieved encouraging specificity (mean in test set: 0.86; 90% empirical CI: 0.66-0.97). Top-performing models across both outcome definitions achieved reasonable overall classifier performance, with area under the receiver operating characteristics curve in the holdout test set of 0.76 and 0.70, respectively.**Conclusions:** While it remains a challenge to predict which patients will experience high-grade NE toxicity, we have achieved good performance in prospectively identifying those patients who are not likely to experience high-grade NE toxicity. Our best-performing classifiers for low-grade toxicity had modest PPV but encouraging specificity, with reasonable overall classifier performance. Our modeling work can support clinicians in prospectively managing patients who may be at risk for toxicity, as well as those patients for whom outpatient monitoring may be desirable.

956 Generation and Characterisation of a Novel Mouse Model for Mucopolysaccharidosis Type IVA

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Mucopolysaccharidosis type IVA (MPSIVA) is a lysosomal storage disease (LSD) caused by mutations in the *Galns* gene with consequent deficiency of N-acetylgalactosamine-6-sulphate sulphatase (GALNS) enzyme. As a result, progressive accumulation of Keratan Sulphate (KS) and Chondroitin-6-Sulphate (C6S) is observed in tissues, clinically translating in severe skeletal phenotypes and growth delay. The enzyme-replacement therapy is the only registered treatment for MPSIVA, though not curative. Based on previous pre-clinical and clinical data obtained for other LSDs in our institute, an *ex-vivo* gene therapy (GT) approach could represent a definitive therapeutic option for MPSIVA patients. However, development of innovative therapies appears challenging since currently available murine models are not sufficiently representative of the human skeletal phenotype. For this reason, we propose to establish and characterise a new MPSIVA mouse model by CRISPR-Cas9 using gRNAs targeting the *Galns* locus in C57BL6/N mice. We obtained five different genotypic variants for *Galns* deletion (*Galns*$^{-/-}$), which are being evaluated using biochemical (enzymatic activity, KS accumulation), morphological (length measurement, weight gain, CT-scan), behavioural (rotarod, grip and footprint tests) and histopathological parameters to select the *Galns*$^{-/-}$ model with the most representative skeletal symptom. Additionally, we have investigated the organ-associated inflammation by monitoring the expression of a panel of inflammatory markers. Biochemical analyses have confirmed all *Galns*$^{-/-}$ to have no Galns activity, whilst KS accumulation has been detected in the peripheral blood of two of our *Galns*$^{-/-}$variants at 4 months of age. However, only one out of four showed clear signs of skeletal disease, mirrored by a reduced weight gain over time and shorter long bones on CT-scan analysis. In line with these results, the same *Galns*$^{-/-}$ variant showed differences in bone resistance and cortical composition on pQ-CT screening and appeared weaker when monitored by a grip-strength test. Preliminary evaluation of organ-specific inflammation has highlighted an upregulation of several inflammatory markers in the spleen of all our *Galns*$^{-/-}$ models. Overall, these results will lead to identify the most representative MPSIVA mouse model for further development of a novel lentiviral-based *ex-vivo* GT, while highlighting cellular and molecular mechanisms behind MPSIVA progression.

957 Adjuvanted Protein Vaccines Boost the Strength and Breadth of the Immune Response Elicited by RNA Vaccine Priming

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Immunological adjuvants commonly used in conventional protein-based vaccines usually target TLRs and other immune sensors to boost the cellular and/or humoral immune response to the vaccine immunogen. These adjuvants represent a powerful but economic and rapid way of boosting vaccine efficacy. With the advent of RNA vaccines and their meteoric rise, the use of adjuvants enhance the impact of RNA and protein vaccines on disease transmission in low-resource environments and by broadening the immune response address the need for constant revaccination to protect against emerging pathogen variants. Four different formulations of a proprietary TLR4-targeting adjuvant (EmT4™; Oil-in-water emulsion, LiT4Q™; Liposomal, MiT4™; Micellar, AlT4™; Alum-adsorbed) were tested in combination with a SARS-CoV2 spike protein, overlapping peptide pools, and RNA to test their effectiveness in enhancing the immune response in mice. In an initial experiment involving a SARS-CoV2 D614G spike protein prime and boost in C57BL6 mice, the addition of any of the TLR4 adjuvant formulations resulted in increased strength of the antibody response against the homologous SARS-CoV2-S immunogen, with the highest IgG endpoint titers reaching up to 2,000,000 by day 56 post-prime, compared to an average titer of around 41,000 for the unadjuvanted regimen. When tested against the more distant Omicron variant (BA.1), the adjuvanted vaccines exhibited higher IgG titers compared to unadjuvanted protein alone, representing an increased breadth of

response. The best performing adjuvant-spike protein combination resulted in a hundred-fold increased average IgG endpoint titer (2.3×10^5) against omicron spike protein compared to the unadjuvanted protein immunization group (2.8×10^3). Pseudovirus neutralization assays showed a similar trend, with adjuvant-protein combinations showing greater neutralization IC50s compared to unadjuvanted spike protein. Three of the four TLR4 adjuvant formulations which performed the best were selected to use in combination with RNA and peptide-based vaccines in a second phase of the study. To test efficacy of boosting with RNA vaccines, adjuvant + protein or adjuvant + peptide pool combinations were used as the boost after a self-amplifying RNA vaccine (saRNA) prime. Initial results show that the inclusion of adjuvant boosted strength in the systemic IgG response against spike proteins of multiple SARS-CoV2 variants including D614G, Delta and Omicron across all modes of immunization. The adjuvants represent a cheap and quickly implementable addition to vaccine formulation, and could be pursued as a cost-effective modification to vaccines in planning for future pandemic responses. Further research in the immunological mechanisms and cellular response to adjuvanted protein, peptide, and RNA vaccinations is warranted to confirm their efficacy and use in preparation for responses to future pandemics.

958 Accelerated Development of Self-Amplifying RNA (saRNA) Vaccine Candidates Using NxGen Microfluidics

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Introduction: The pandemic of COVID-19 remains challenging to control due to progressive emergence of multiple variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), despite the availability of several clinically approved vaccines. However, multiple booster doses of existing clinically approved conventional mRNA-based vaccines for human use have shown progress in producing protective immunogenicity against the variants of SARS-CoV-2. Self-amplifying mRNA has emerged as a newly designed mRNA platform that can replicate itself, allowing substantially lower dose for effective immune response. Herein, we demonstrate the preclinical success of saRNA-based vaccines encapsulated in lipid nanoparticles (LNPs), and lab scale manufacturing to GMP scale using microfluidic technology and the immune response against emerging variants of SARS-CoV-2. **Methods:** Novel ionizable lipids were selected from a proprietary lipid library after assessing the physicochemical profiles and biological activity for its use in LNP. The saRNAs were synthesized from in vitro transcription. Full-length SARS-CoV-2 spike protein-encoded saRNA-encapsulated LNPs were manufactured using NxGenTM microfluidic NanoAssemblr technology platform and characterized for hydrodynamic size, polydispersity index (PDI), loading, encapsulation efficiency, and long-term storage stability. The lead saRNA-based vaccine candidates were screened and assessed for protective immunogenic responses including humoral and cell-mediated immunity, following a prime-boost with 4 weeks apart in various animal models. Protective immunogenic responses of saRNA-based vaccine candidates were evaluated in a hamster virus challenge model against SARS-CoV-2 virus. The vaccine candidates were then tested in Rhesus Macaques. Viral neutralization titres for both Delta and Omicron variants were further analyzed. **Results:** saRNAs-encapsulated LNPs produced were in the desired size range of 60- 80 nm with a PDI value below 0.2 and with an encapsulation efficiency over 95%. In respect to both in vivo efficacy and physicochemical properties, neither batch-to-batch variations nor instability of our lead vaccine candidate were identified. Our repeated preclinical investigations exhibited the generation of robust humoral and cell-mediated immune response in both mouse and hamster models. In both animal models, no clinical symptoms or adverse effects were observed. Moreover, the obtained sera of vaccinated mice showed protective coverage for both Delta and Omicron variants. The lead vaccine candidates protected the hamsters from weight loss and an increased lung weight following SARS-COV-2 challenge. Vaccinated hamsters had higher levels of SARS-CoV-2 specific IgG antibodies and reduced viral loads. The immune response seen in small animal models were translated to NHP. **Conclusion:** Overall data provide evidence for safety and efficacy of saRNA based vaccines, and for accelerated development of bench to large scale manufacture of saRNA LNP vaccine candidates using NxGenTM microfluidic technology platform. NxGen platform is fully capable of scaling-up large volumes of LNP-based saRNAs vaccines while retaining Critical Quality Attributes (CQA) of the drug product. In addition, the obtained protective immunity in various animal models from our preclinical studies strongly highlight the value for development of saRNA LNP based vaccines along with mRNA LNPs to be used effectively during a pandemic response.

959 Brain-Directed AAV Gene Therapy Corrects Lethal Neurodegeneration & Improves Locomotor Behaviour in a Mouse Model of CLN5 Batten Disease

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The neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease, are a group of inherited lethal neurodegenerative lysosomal storage disorders. CLN5 disease is caused by mutations in the *CLN5* gene encoding a soluble lysosomal lumen protein with cysteine-based *S*-depalmitoylase activity. Children with CLN5 disease suffer progressive motor dysfunctions, vision loss, seizures and dementia, eventually leading to premature death. It is currently incurable and there is a desperate need for a novel effective therapy. Here we carried out a preclinical study of an adeno-associated virus (AAV) - mediated gene therapy in a transgenic mouse model of CLN5 disease, with comparison of therapeutic efficacy between two different promoters used to control *CLN5* expression.

A single dose of AAV9 vectors carrying the human *CLN5* gene driven by either the CAG or the synapsin promoter was administered via intracerebroventricular (ICV) injecton into neonatal Cln5 mice. Treatment efficacy was evaluated by assessment of neurodegeneration and monitoring of locomotor functions and life span. In a second trial, single-dose AAV9.hCLN5 was delivered ICV into juvenile early-symptomatic Cln5 mice at 4 weeks of age, to assess treatment efficacy of later stage gene therapy intervention in CLN5 disease. Neonatal ICV administration of AAV9 expressing human *CLN5* driven by the neuronal specific synapsin promoter significantly prevented neurodegeneration, improved long-term locomotor functions and extended lifespan of the Cln5 mice. However, such brain-directed gene transfer employing the ubiquitous CAG promoter demonstrated limited therapeutic efficacy. The vector carrying *CLN5* driven by the synapsin promoter showed enhanced efficacy when delivered at a juvenile early-symptomatic stage, also resulting in long-term disease attenuation, which provides a particularly practical case for clinical translation. These results indicate that brain-directed AAV gene therapy can be a promising treatment strategy for CLN5 disease.

960 Developing an AAV-Based Gene Replacement Therapy for Mitochondrial Alanyl-tRNA Synthetase 2 (*AARS2*) Leukodystrophy

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The nuclear *AARS2* gene encodes mitochondrial alanyl-tRNA synthetase 2 that is responsible for attaching alanine onto the cognate tRNA in mitochondria. The resulting alanyl-tRNA participates in mitochondrial translation that produces the key proteins constituting the mitochondrial respiratory complexes. *AARS2* mutations have been implicated in two recessive diseases: infantile cardiomyopathy and late-onset leukodystrophy. Currently, there is no treatment available for *AARS2* related diseases. To develop a potential gene replacement therapy, we generated recombinant AAV9 vectors expressing human *AARS2* driven by a ubiquitous promoter (rAAV9.hAARS2), and injected wild-type (WT) mice to test safety of AARS2 over-expression. We found that systemic delivery to neonatal mice via facial vein injection caused animal death within a week, whereas intracerebroventricular injection was well tolerated. TUNEL assay revealed apoptotic hepatocytes in the animals receiving facial vein treatment, suggesting liver toxicity. Therefore, we modified the expression cassette to include miR122 binding sites that enable liver-specific, miR122-mediated transgene silencing (rAAV9.hAARS2.miR122BS). Indeed, the modified vector design prevented hepatocyte apoptosis and animal lethality following facial vein delivery. To characterize the functional consequences of AARS2 malfunction and establish meaningful biochemical readouts of AARS2 restoration, we obtained fibroblasts from a 16-year-old patient diagnosed with leukodystrophy and carrying compound heterozygous *AARS2* mutations. To our surprise, mitochondrial alanine tRNA charging efficiency was not compromised and mitochondrial function was intact as determined by a deep sequencing-based assay and Seahorse assay, respectively. We have reprogrammed the patient fibroblasts into induced pluripotent stem cells (iPSCs), and are in the process of generating iPSC-derived neurons and oligodendrocytes to study potential cellular defects in the disease-relevant cell types. Furthermore, to generate a mouse model of *AARS2* disease, we knocked in a patient-relevant mutation into mouse *Aars2* gene and obtained chimeric founders. The mutant allele is being tested for germline transmission. Together, our study supports the safety of brain-directed *AARS2* gene replacement therapy and calls for a deeper understanding of the disease pathomechanisms in suitable cellular and animal models. (#Co-corresponding authors)

961 *In Vivo* HSC Gene Therapy Using BaEVRLess-Pseudotyped Retroviral Vectors

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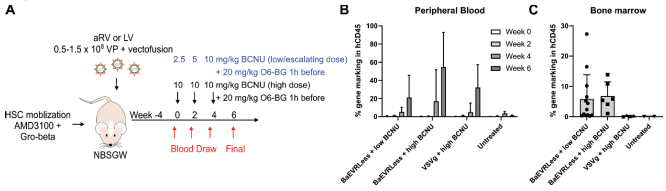
In vivo hematopoietic stem cell (HSC) gene therapy has several potential advantages over *ex vivo* gene therapy, such as eliminating the need for stem cell harvest, *ex vivo* manipulation of cells and conditioning of the patient as well as potentially reducing costs. The VSVg envelope glycoprotein is commonly used for pseudotyping of retroviral vectors but is not well suited for *in vivo* application due to its serum sensitivity and poor ability to mediate gene transfer into quiescent HSCs. In contrast, the baboon endogenous retrovirus (BaEV) glycoprotein and its derivative BaEVRLess allow efficient gene transfer into resting HSCs and are serum resistant. The aim of this work is to explore the potential of BaEVRLess-pseudotyped retroviral particles for *in vivo* HSC gene therapy (Fig. A). Competitive experiments revealed that both alpha-retroviral and lentiviral particles can transduce CD34+ cells at similar rates *in vivo* and are suitable for *in vivo* application. To enhance gene transfer, we first optimized the mobilization of HSCs in humanized NBSGW mice. The combination of AMD3100 and Gro-beta efficiently mobilizes HSCs within 60 minutes of drug application in a range of 3-112 hCD34+ cells/μL of blood. 0.5-1 x 10^8 viral particles were injected intravenously into animals after mobilization. Initial gene marking reached up to 3% of hCD45+ cells three days post injection and stabilized around 1% four weeks after *in vivo* transduction. To select for gene marking, we overexpressed MGMT-P140K in transduced cells, which mediates resistance to the alkylating agent BCNU. After three cycles of BCNU treatment, we observed a 20-fold (0.7% to 21.1% GFP+) or 144-fold (0.38 to 54.6% GFP+) enrichment of gene marked cells in the peripheral blood using either a low or a high dose regimen, respectively (Fig. B). In the bone marrow, gene marking reached 5.8% in the low-dose group and 6.8% in the high-dose group (Fig. C). Transduced cells were present in lymphoid and myeloid lineages. We detected 18.1% gene-marked hCD34+ cells in the bone marrow after engraftment. Finally, we combined the MGMT-P140K overexpression with a therapeutic payload by targeting the

transcription factors BCL11A and ZNF410 using miRNA-embedded shRNAs (shmiR) containing vectors. Downregulation of BCL11A and ZNF410 in erythroid cells leads to sustained reactivation of gamma-globin and induction of fetal hemoglobin, which largely attenuates the hematologic effects of sickle cell disease. Following *in vivo* gene transfer and BCNU selection in NBSGW humanized mice, the bone marrow cells were harvested for *in vitro* erythroid differentiation and enrichment of transduced cells via FACS. We observed a significant upregulation of gamma-globin (35.6%) and fetal hemoglobin (25.1%). In summary, we demonstrate the proof-of-principle that BaEVRLess-pseudotyped retroviral particles can be applied for *in vivo* gene therapy, e.g. to treat sickle cell disease.



962 GMP Compliant BACS Microbubble Bag-Based T Cell Isolation in a Closed System CAR-T Manufacturing Workflow

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In order to treat complex diseases such as cancer, scientists developed treatments based on immune cells that have been modified to improve their effector functions, fine-tune their specificity, increase their ability to survive long-term after adoptive transfer, or a combination thereof. Chimeric antigen receptor (CAR-T) cells represent one version of this. An important early step in the manufacturing of CAR-T cell-based therapies involves the isolation of purified T cells from patient leukapheresis material. Traditionally, this has been accomplished using iron particles and cumbersome magnetic separators. While these methods have a demonstrated utility in the manufacturing of cellular therapies, they have proven difficult to scale and pose a significant financial burden on development programs. In order for the curative potential of cellular therapeutics to be fully realized by patients, disruptive technologies that enable increased manufacturing scale, decreased manufacturing time, and cut overall costs down are vital. Here we present a method for inserting buoyancy activated cell sorting (BACS™) microbubbles into common closed-system CAR-T manufacturing workflows and demonstrate the compatibility of our GMP grade reagent with automated cell processing instruments like the Rotea counterflow centrifugation system. First, leukapheresis material is passed through the Rotea system for washing and platelet removal. Second, an in-bag negative selection of T cells is performed using a novel BACS microbubble cell isolation protocol. Finally, untouched, negatively selected T cells are transferred to a new bag as part of a buffer/media exchange using the Rotea and the isolated cells are then carried through the remainder of the CAR-T manufacturing workflow. This study demonstrates the seamless incorporation of GMP grade BACS microbubbles into existing closed-system workflows without disruption of current manufacturing processes. Using buoyant microbubbles for cell separation allows for quick and gentle isolation of cells, resulting in consistent performance over a wide range of starting leukopak cell compositions. In addition, BACS microbubble isolation yields more purified T cells than traditional methods, and does so in a fraction of the time. The use of next generation microbubble cell isolation enables higher throughput and offers a truly scalable solution to cell isolation. Disruptive technologies such as BACS microbubbles will aid in increased access to cutting-edge cellular therapies for patients across the globe.

963 Novel AAV Constructs for Targeted ApoE2 Delivery Counteracts the Impact of ApoE4 Expression in Amyloid Deposition and Neuroinflammation in Mouse Model of Amyloidosis

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Dementia and more specifically Alzheimer's Disease (AD) pose a global health crisis with more than 3 million cases of AD in the US alone. Inheritance of the ε4 allele of apolipoprotein E (ApoE) is the strongest genetic risk factor associated with the sporadic form of AD, whereas the rare ApoEε2 allele has the opposite effect. However, the mechanisms whereby ApoE confers risk and protection remain uncertain. The ApoEε4 genotype is associated with worsening plaque deposition, increased speed of cognitive decline and increased neuroinflammatory microglia phenotypes. We previously showed that ApoEε4 or ApoEε2 secretion from ependyma after AAV delivery to amyloid plaque-bearing transgenic mice (expressing wild type murine apoE) exacerbated or reduced amyloid plaque burden respectively (Hudry 2013 Sci Trans Med). Here we advance our AAV based gene transfer approach using a novel capsid coupled to a promoter which restricts ApoE2 expression to ependymal cells. When delivered to plaque-bearing mice expressing human ApoE4, and devoid of mouse *apoe* (APP/PS1/mApoE$^{-/-}$/hAPOE4$^{+/+}$) ApoE2 reduced the size and density of cortical amyloid plaques and the concentration of oligomeric Aβ in the brains of these animals. Additionally, we observed reduced microglia activation near plaques indicating that ApoEε4 may prime microglia towards an inflammatory phenotype and that exogenous ApoE2 interrupts or reduces this aberrant activation. Our data support that focused expression of ApoE2 into CSF in ApoE4 carriers may positively impact the classical lesions of AD (eg plaque deposition) as well as the increased neuroinflammatory profile observed in sporadic AD.

964 A High-Throughput Method for the Testing and Optimization of CAR-T Multi-Targeting Approaches

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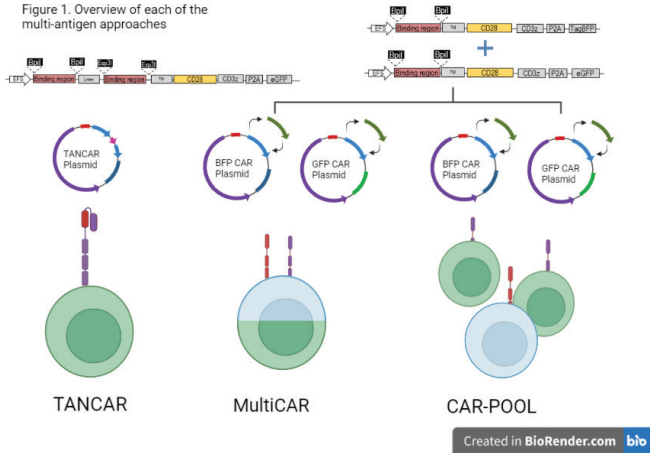
Chimeric antigen receptors (CAR) are fusion proteins which reprogram immune cells to target a specific target antigen when transduced. CAR expressing T-cells (CAR-T) have emerged as an effective treatment method for hematological cancers; however, safely targeting solid tumors has been more difficult. While on target, off tumor toxicity is treatable for hematological cancers, solid tumor antigens overlap with healthy tissue; therefore, requires more precise targeting in order to safely treat patients. Single targeting CAR-T therapy can also be rendered ineffective by tumor mutations such as antigen escape or internalization. By targeting multiple antigens, it is thought that safer and more functional CAR-T therapies can be achieved, but identifying the right combination of antigens and the most optimal combinatorial molecular strategy is a formidable challenge. Here we present the first step in a high-throughput method to screen and optimize multi-antigen CAR strategies. Previously we created modularized CAR plasmids to allow quick and easy recombination of antigen binding domains (ABDs) to create and screen new CAR molecules; here, we sought to extend that platform to screening of multi-antigen targeted CAR molecules. We created a simple set of tools for screening three distinct multi-antigen CAR strategies: Tandem (Two binders, one body), Dual (Two complete CAR proteins on a single cell) and Pool (Two single CARs administered simultaneously or sequentially) and screening their functional activity in high throughput. An overview of this can be seen in figure 1. As proof-of-concept we screened novel binding domains for multi-antigen targeting of hematological malignancies (CD22 and BCMA) or various solid tumors (EGFR and Mesothelin). We found surprisingly variable target responses and tonic activity profiles depending on the specific combination of the ABDs and combinatorial method used. The proof of concept also demonstrated that CAR signaling was maintained though a simulated antigen escape using CRISPR knock-out cells. In primary T cells where differences were amplified, we found that multi-CARs generated through co-transduction consistently outperformed tandem-CAR molecules. In summary, Multi-CAR-J and the related plasmids provide an easy, high-throughput method to assess and compare different methods of combining CARs allowing for an optimal combination of binders with the correct multi-CAR format. We hope to expand this platform from screening to customization, allowing for the future implementation of strategies such as Boolean logic gates, for a safer, more specific CAR treatment.

Figure 1. Overview of each of the multi-antigen approaches



965 Investigating the Kinetics of Prime Editing

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Prime Editing (PE) adds a new level of precision to CRISPR based gene editing. It enables efficient search and replace of specific DNA sections without templates or indels and with lower off-target effects. Research in this field currently focuses on optimization of editing efficiency. Although highly optimized PE protocols exist, little regard has been paid to the role of delivery timing and kinetics of Cas9 mediated editing. A good understanding of PE-kinetics is desirable for applications were quantitative control is required including future gene therapies or PE based biocomputing. In our work, we address the question, how the kinetics of PE depends on defined changes in the system, for example, delivery vehicles, vectors or pegRNA variations. To this end, single cell readout is advantageous. As demonstrated in this contribution Live-Cell Imaging on Single Cell Arrays (LISCA, Fig. 1), enables time-resolved tracking of edit events at the single cell level.

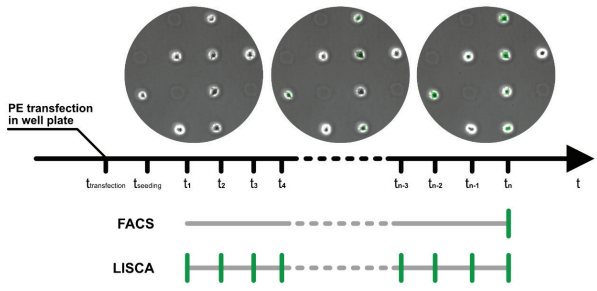

Figure 1: *In our experiments, we measure the fluorescence of single HEK293T cells on a pattern every 15 minutes over 48h, which, compared to the commonly used single time point FACS measurements, gives us a better understanding of PE kinematics.*

As a model reporter system, we use HEK293T cells, which stably express a blue-shifted mGreenLantern (bs-mGL) protein. Only two

base edits in close proximity, i.e. on one pegRNA, are required to change it back to the original mGreenLantern (mGL) sequence. Plasmid transfection of the PE system enables the cells to perform these edits and then express the original mGL protein again. To prepare our experiments, cells are seeded and grown overnight in well plates. They are then co-transfected with both PE2 and a corresponding pegRNA as separate plasmids and incubated until the recommended change of media period has passed. Afterwards, we transfer them to a slide that was patterned for single cell adherence. After an additional incubation period, the slide is loaded on to a fluorescence microscope. Using LISCA, we can then track the green fluorescence signal of single cells over an extended period. This allows us to compare the timing of different pegRNAs, which only differ in actual edit length - first edit to last edit - inside the Reverse Transcriptase template. Using LISCA, we compared the onset times of these pegRNAs, which showed a significant difference (Mann-Whitney-U test, p-value = 1.054e-08, W=1133) during the first 48h, while the FACS measurements interestingly showed them to have a similar efficacy. These differences highlight how pegRNA variations can affect the timing of PE and warrant further studies to investigate similar effects. Gaining a deeper understanding of the kinematics can help us achieve more controlled PE in the future.

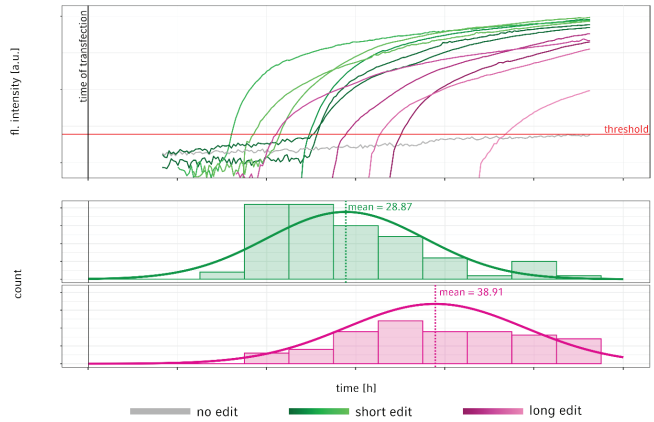

Figure 2: *Fluorescence time courses of single cells over 48h. (1) Fluorescence signal integrated over cell area for presented in logarithmic scale. We define the onset time as the time where fluorescence exceeds the threshold level as indicated. (2) The distribution of on-set times shows a clear shift towards faster editing of short edits compared to long edits.*

966 A Novel Vector for Non-Viral Gene Therapy of Hemophilia A

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Rare genetic diseases such as Hemophilia are well suited for the gene therapy strategies. First, the expression of clotting factor VIII (FVIII) and factor IX (FIX) gene is not tightly controlled and second, a minimal increase in circulating proteins, even as low as 5% of normal physiological levels, can improve the clinical phenotypes. The current standard of care for Hemophilia A is prophylaxis aimed at increasing FVIII levels to a sufficient level to prevent bleeding episodes and reduce the occurrence of hemarthrosis. Though effective the currently available treatments have limitations of short therapeutic half-life and frequent intravenous infusions, which invokes the development of gene therapy strategies. Recombinant adeno-associated viral (rAAV) vectors are the predominant gene therapy vectors used for several genetic diseases including Hemgenix®, recently approved gene therapy treatment for Hemophilia B patients. However, AAV-mediated gene therapy has several limitations such as pre-existing neutralizing antibody, inability to treat pediatric patients, and a limited cargo capacity, which confines the use of AAV for genetic diseases with a smaller (<5 kb) transgene. Recent advances in vector technology have made the non-viral gene therapy approaches particularly appealing for the chronic diseases such as Hemophilia. The non-viral vectors could potentially circumvent the limitations associated with rAAV vectors and provides the flexibility of inserting a large transgene such as human FVIII together with the regulatory elements such as liver-specific promoter, intron, and transcriptional enhancer for the long-term persistent expression. Typically, non-viral gene therapy uses a plasmid DNA to transfer the transgene of interest into recipient cells. However, the plasmid DNA made in the bacterial system normally contains an antibiotic resistance gene and prokaryotic origin of replication, which potentially triggers an innate immune response upon gene transfer. Besides, there is a significant level of endotoxin contamination in the plasmid DNA prep made from the bacterial system, which could reduce the gene transfer efficiency. Ideally, the DNA for non-viral gene therapy should contain a transgene of interest and lack prokaryotic extraneous sequences as well as endotoxin contamination. The baculovirus-insect cell system (BICS) is a eukaryotic cell-based system that makes it a suitable platform to produce DNA vectors for non-viral gene therapy. In this study to be presented, we utilized the BICS to develop a novel platform technology including, a versatile baculovirus shuttle vector "BIVVBac" bacmid, for non-viral vector production in a form of closed-end DNA (ceDNA). Unlike encapsidated rAAV vector genomes, the ceDNA vector has no packaging constraints imposed by the limiting space within the viral capsid. In this study to be presented, we demonstrated the feasibility of BIVVBac system for non-viral vector (ceDNA) production by using human coagulation factor VIII (hFVIII) together with the regulatory elements such as liver-specific promoter, intron, transcriptional enhancer, and XTEN to enhance the circulating half-life in Hemophilia A patients. Furthermore, we explored the efficacy of several parvoviral ITRs in driving long-term persistence expression of FVIIIXTEN in Hemophilia A mice. The results indicated that the FVIIIXTEN ceDNA persistently produce up to 200% of normal physiological level of FVIII for more than 6 months at 20x lower the dose of other DNA forms tested by the systemic administration in Hemophilia A mice model. Thus, in this study to be presented, we demonstrated the feasibility of our novel vector (ceDNA) for the non-viral gene therapy of Hemophilia A.

967 A Rapid Method with Wide Dynamic Range to Assess Size-Based Purity and Concentration of AAV5 Particles by Size-Exclusion High Performance Liquid Chromatography

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Background: Monitoring of critical quality attributes (CQAs) is essential to ensure that high quality and well-characterized biological products are manufactured consistently. Size exclusion chromatography (SEC-HPLC) is a rapid analysis method for viral particle quantification that separates them based on their hydrodynamic volume, which generally correlates with size. This allows separation of intact virus particles such as adeno-associated virus (AAV) from other cellular contaminants and fragmented or aggregated virus particles. However, current standard SEC-HPLC methods for virus particles typically have a relatively long run time per injection, a narrow dynamic range, and require high amounts of virus per injection. Here we present a rapid method to adequately quantify monomeric and multimeric AAV particles by SEC-HPLC over a range of four log scales. **Methods:** AAV5 particles were generated internally in HEK293 cells. The capsid titer was determined using Progen AAV5 titration ELISA kit following manufacturer's instructions. ELISA titer results were verified by particle concentration measurement with dynamic light scattering (DLS) using a Malvern Panalytic Zetasizer Ultra Red. Analytical size exclusion high performance liquid chromatography (SEC-HPLC) was performed on a Waters Arc Premier HPLC system PDA and FLR detector. Separation was monitored at 215 nm, 280 nm, and intrinsic fluorescence (ex 280 nm / em 348 nm). A novel Phenomenex Diol SEC column (4.6 x 150 mm, 3 µm) was used at a column temperature of 25°C. Isocratic elution was performed with 20 mM sodium phosphate, 350 mM potassium chloride, pH 6.6. Sample temperature was maintained at 5°C. Samples were diluted formulation buffer. Injection volumes were between 1 and 50 µL. **Results:** Capsid titer of the purified AAV5 particles was determined by ELISA as 2.10E+14 vp/mL. This value was confirmed by an orthogonal DLS. Size-based separation of AAV5 particles was monitored at two wavelengths for UV absorbance and intrinsic fluorescence. Each of these channels monitored has its own range where data was found linear, precise, and accurate (Figure 1). USP resolution between monomeric and multimeric AAV peak was between 1.7 and 1.8 for all injections within the linear range. The percentage of monomeric and multimeric particles were calculated based on the relative percent area of the integrated peaks in each channel. For titer of viral particles, the number of viral particles per milliliter was quantitated from an AAV standard at a predetermined titer. The number of multimeric particles was consistent at 5% over the entire range of the method. **Conclusion:** The method presented here has a significantly improved separation time compared to traditionally used methods (7 min versus 20-30 min per injection). Monitoring of intrinsic fluorescence increases sensitivity over UV detection of proteins. The use of intrinsic fluorescence as well as ultraviolet absorption at 215 nm and 280 nm provides a four-log dynamic range for this method ranging from 5.25E+08 to 5.25E+12 virus particles column load. This HPLC method with multiple monitors creates an opportunity to improve the quality of information to guide development, validation, and monitoring of production processes for AAV virus particles.

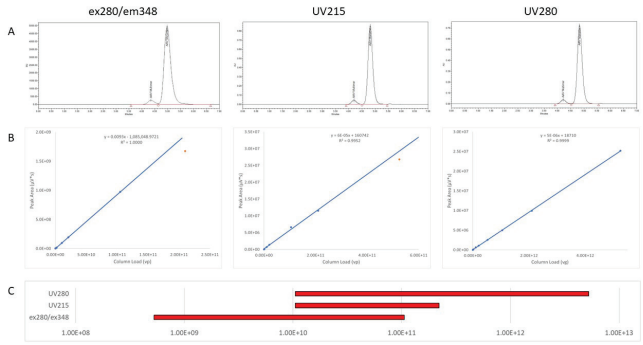

Figure 1: (A) Example chromatograms and (B) linear range at ex280/em348, UV215, and UV280. (C) Detection range of the method.

968 *In Vivo* Time-Course and Tissue-Wide Gene Profiling Reveals Distinct Signatures of CAR-T and CAR-iNKT Cell Responses to Solid Tumors

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Chimeric antigen receptor T (CAR-T) cell therapy has shown remarkable success in treating blood cancers but only limited efficacy against solid tumors. Lack of knowledge of in vivo behaviors of CAR-T cells, especially in the complex solid tumor microenvironments, is considered as a major hinder hampering the successfully application of CAR-T cell therapy for treating solid tumors. In recent years, unconventional T cells, such as invariant natural killer T (iNKT) cells, have attracted increasing attention as alternative CAR-mediated cell therapy carriers, due to their strong tumor killing capacity, promising solid tumor homing ability, as well as their potential for developing "off-the-shelf" cell therapy. iNKT cells exist in extremely low numbers (0.001-1%) in health donor blood, that greatly limits their clinical applications. To overcome this critical limitation, our lab has developed a new technology to produce therapeutical levels of CAR-iNKT cells through a stem-cell engineering and iNKT differentiation cell culture strategy. Here, we report a study to compare the conventional CAR-T cells with the alternative unconventional CAR-iNKT cells, aiming to obtain an extensive understanding about their in vivo spatial and temporal behaviors against solid tumors. Mesothelium (MSLN) targeting CAR-T and CAR-iNKT cells were administered into NSG mice bearing OVCAR3 human ovarian tumor xenograft. Overtimes, tissues were collected from tumor site and other sites (peripheral blood, spleen and liver); single cells were isolated and subjected to single-cell RNA sequencing (scRNA-seq) analysis. Cells analyzed include the therapeutical cells (CAR-T and CAR-iNKT cells), tumor cells, and tumor microenvironments (TME) cells. Our results revealed several findings. 1) Both CAR-T and CAR-iNKT cells exhibited dynamic gene profiling changes, starting by acquiring an effector/proliferating signature, and gradually transition to progenitor/terminally exhausted gene profiles. These changes happened across all tissues examined, with the changes at tumor site leading the changes at other tissues. 2)

In contrast to CAR-T cells, CAR-iNKT cells displayed significantly enhanced gene profiles that correlated with their stronger in vivo anti-tumor efficacy. 3) CAR-T cells induced immune evasion in tumor site—loss of antigens, while CAR-iNKT cells did not; likely due to their multi-targeting mechanism. 4) CAR-T cells triggered an immunosuppressive and cytokine release syndrome (CRS)-prone TEM while CAR-iNKT cells actively altered TME and reduced CRS gene signatures by eliminating tumor associated macrophages (TAMs). Collectively, our result provides the first-time insight into the in vivo spatial and temporal behaviors of two important cancer cell therapy platforms CAR-T and CAR-iNKT cells. Such knowledge will be valuable for guiding and selecting of proper cell therapy products, and further improving these cell therapies for solid tumors.

969 Abundant Expression of IFNγ by GAIA-102, an Off-the Shelf and Highly Activated NK-Like Cell Product, Induces MHC Class I Expression

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Background: We previously established an original method to generate highly activated NK-like cells(GAIA-102) from mixed peripheral blood mononuclear cells from multiple donors. Now three Phase I clinical trials, including neuroblastoma, for assessing the safety and potential anti-tumor activity are ongoing. In the preclinical studies, we found thatGAIA-102 demonstrated the 2-independent anti-tumor mechanism *in vivo,* namely, the induction of not only efficient mass-reduction but alsothe establishment of anti-tumor acquired immunity *in vivo*; however, theunderlying mechanism is still unclear. Malignant tumors escape from $CD8^+$T cell attack through loss of MHC class I (MHC-I) expression, therefore MHC-I upregulation is important for inducing tumor-specificacquired immunity. The purpose of this study, therefore, was to evaluatethe activity of GAIA-102 to upregulate the tumor MHC-I. **Methods:** GAIA-102 was dissolved in Plasma Lyte-A (a clinically available drip infusion) and cocultured with or without K562 cells (CML cell line), and the supernatant was collected. SK-N-SH cells (human neuroblastoma cell line) were treated with the supernatant, followed by surface MHC-I detection using flow cytometry. **Results:** Both the supernatant of GAIA-102 only as well as K562 and GAIA-102 coculture upregulated MHC-I expression of SK-N-SH cells, as increased maximally by 14 folds compared with control. Such effect was diminished in the use of boiled supernatant, suggesting the factor might be a secreted protein, and this effect was completely abolished by IFNγ neutralizing antibodies. The IFNγ concentration in the supernatant of GAIA-102 at 2, 6, and 24 h were 13.0,32.0, and 72.6pg/ml, respectively, and those in the supernatants cocultured with K562 showed approximately 3-fold higherexpression. **Conclusion:** We clarified that GAIA-102 could upregulate the MHC-I expression of tumor cells, by increasing IFNγ secretion in response to tumor cells. We concluded that GAIA-102 might enhance acquired immunity through IFNγ-mediated mechanism.

970 The Retargeted Universal Fusosome: A Modular Approach to Generate Fusosomes for Targeted Gene Delivery

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In vivo targeted gene delivery has the potential to significantly improve the efficiency and safety of gene therapy for patients. We have previously shown that our gene therapy platform using a retargeted viral vector (fusosome) enables efficient and cell-specific gene delivery to a variety of cell types both *in vitro* and *in vivo*. The fusosome is an integrating viral vector pseudotyped with two viral glycoproteins (that act as a single fusogen) with distinct attachment and fusion functions. The attachment fusogen (G protein) is blinded to its native targets and engineered by fusing a ligand that binds a receptor that is specifically enriched on the surface of a given cell type to generate a G-binder fusion protein. Interaction between this retargeted G protein and its receptor triggers the F protein to mediate membrane fusion resulting in gene transfer from the viral vector to the target cell. Using this approach, we and others have successfully targeted several cell types including hepatocytes and different T cell subpopulations. While this approach works well, our current method requires cloning, expression, and screening of hundreds of different G-binder fusion proteins to identify a few that function well as fusogens in a fusosome. Fusing a binder to the G protein can decrease its surface expression and incorporation into the membrane of the viral vector, potentially disrupting G conformation and functionality, and ultimately reducing the infectivity of the viral particles. Each binder can perturb one or more of these parameters in a unique way requiring extensive screening and optimization to evaluate and identify a lead candidate for each target. To overcome these limitations, we have leveraged a modular approach which eliminates the need for the fusosome production cells to express the G-binder fusion protein; instead, the binder is coupled to the G protein *after* the production of the "Universal Fusosome." This is enabled by addition of a small peptide (adapter) to the blinded G protein. The retargeting binder is fused to a complementary protein that can bind and react with the adapter resulting in an irreversible conjugation. The adapter has been chosen and optimized such that the level of expression and incorporation of the blinded G protein into the viral particles is comparable to that seen for viral vectors pseudotyped with wild type G protein. This design results in a modular system in which retargeting binders can be covalently added to a Universal Fusosome with G levels independent of the binder itself. Here, we present proof of concept data showing efficient and specific gene delivery to a target cell of interest using fusosomes produced by this novel approach. In addition, we demonstrate that this Universal Fusosome coupling approach rescued the suboptimal infectivity observed for several binders when the latter were tested as fusogens using the standard single G-binder fusion protein method. This retargeted Universal Fusosome is a modular system for producing fusosomes for cell-specific delivery. It has the potential to be more time- and cost-effective, removing the need for extensive screening process to identify lead candidates for each target receptor/cell of interest.

971 A Novel Vector for CAR-T Cells with Enhanced Antitumor Response and Reduced Risk of CRS and ICANS

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CAR-T cell therapies have shown effective antitumor response against hematological malignancies, and several CAR-T cell products targeting CD19 or BCMA have already been approved. However, since relapse occurs frequently and the therapeutic effect on solid tumors is limited, further development of CAR-T cells with long-lasting antitumor response is required. On the other hand, suppression of side effects must also be considered. Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) are the most frequent side effects, and enhancing the antitumor effect could lead to an increase in these risks. Therefore, it is necessary to develop CAR-T cells with high antitumor response while suppressing serious side effects. Recently, we have developed a novel cytokine receptor that captures IL-6 and IL-1, which cause CRS and ICANS, respectively. Expression of the novel cytokine receptors in CAR-T cells is expected to reduce side effects due to absorption of cytokines secreted by macrophages. In our novel cytokine receptor, we used the extracellular domain of IL-6 receptor alpha chain fused to domains of GP130 to increase the affinity to IL-6, and linked the transmembrane and intracellular domain of the mutated IL-7 receptor alpha chain, which constitutively activates the JKA/STAT pathway while attenuating Akt signaling. Co-introduction of CAR and the chimeric cytokine receptor using individual viral vectors has resulted in long-term in vivo persistence of CAR-T cells and improved therapeutic efficacy. In addition, IL-1 Receptor Type 2 was co-expressed to absorb IL-1 beta in CAR-T cells, and the chimeric cytokine receptor-expressing CAR-T cells could efficiently reduce both IL-6 and IL-1. In order to apply this technology to the clinic, it is necessary to develop a single vector that can highly express individual genes in terms of therapeutic efficacy and cost efficiency. In this study, we have developed all-in-one lentiviral vector to improve and optimize the expression level of each gene. First, we generated lentiviral vectors with various internal promoters and selected the MND promoter as a suitable promoter for CAR-T cells. We then enhanced expression level and function of receptors by codon optimization. When expressing multiple factors in a single vector, the expression balance of each factor is important. Vectors were constructed by switching the order of CAR, IL-6 chimeric receptor, and IL-1 receptor to determine the most functional design. Furthermore, we have confirmed that insertion of woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in this vector enhanced its function. T cells transduced with the all-in-one vector combining these approaches were used to evaluate therapeutic efficacy in a solid tumor mouse model, and showed significantly enhanced T cell survival compared to standard CAR-T cells, leading to almost complete tumor regression. As a versatile CAR-T platform, this technology promises to be widely applicable regardless of the target antigen to enhance its efficacy and safety.

972 Identification of Novel Mutations in PCCA and PCCB Genes in Fibroblasts from Patients with Propionic Acidemia via Long-Read Sequencing

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Propionic Acidemia (PA) is a rare autosomal recessive inherited metabolic disease caused by a deficiency of propionyl-CoA carboxylase (PCC) activity. PCC is a biotin-dependent, dodecameric mitochondrial enzyme comprised of six α (PCCA) and six β (PCCB) subunits that catalyzes the carboxylation of propionyl-CoA to methylmalonyl-CoA. Mutations in either gene can cause PA, which manifests with allelic and genetic heterogeneity throughout both PCCA and PCCB genomic sequences. Fibroblasts from patients with PA (PA fibroblasts) were obtained from the Coriell Institute for Medical Research, which maintains biobanks of patient cells for many diseases for medical research. Unfortunately, for most of these PA fibroblasts there is no available information on the underlying genetic mutations. Here, we used targeted long-read DNA sequencing to identify genomic mutations. We followed-up with PCR-enriched long-read RNA sequencing to confirm the haplotype and suspected splice variants in all eight available cell lines of fibroblast from patients with PA and two unaffected parent fibroblast cell lines. We found that five PA fibroblasts harbored mutations in the PCCA gene and three possessed PCCB mutations. Consistent with the known genetic complexity of PA inheritance, six patients harbored compound heterozygous and two had homozygous mutations. The two parent fibroblast lines were heterozygous for PCCA, and the associated mutations were manifested as compound heterozygous in fibroblasts from their child with PA. Another PA fibroblast line possessed mutations in both genes, ultimately determined as compound heterozygous PCCA and monoallelic PCCB. In total, out of 14 unique disease-associated variants, 10 mutations were located within exons and four mutations led to splicing variants. A comprehensive search of dbSNP, ClinVar and PubMed databases suggested that our analysis identified four unreported PCCA genetic variants, and one unreported PCCB genetic variant associated with PA. This information will be helpful for researchers and clinicians diagnosing patients with PA, utilizing these PA fibroblasts, and developing therapeutics for PA. Furthermore, our experimental approach could be adapted to characterize other diseases with complex underlying genetic mutations.

973 Evaluation of the Immunoregulatory Effects of Mesenchymal Stem Cell Derived Extracellular Vesicles

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Introduction. Novel investigations in regenerative research highlight the in vivo and in vitro immunoregulatory properties of Mesenchymal

Stem Cell's secretome attributed to Extracellular Vesicles (MSCs-EVs). EVs is a heterogenous population of cell - free double layer membrane - bound carriers enriched with parental cell-derived active cargos that contribute to the intracellular communication through the delivery of signal molecules. More specifically, stem cell derived EVs have been proved to alleviate neuroinflammation and neurodegeneration via suppression of astrogliosis and microgliosis. In addition, they can also cross the brain - blood barrier, making them a promising tool to fight the CNS pathology. Sandhoff disease (SD) is a GM2 Lysosomal storage disease (LSD), caused by specific mutations of the N-acetyl-β-hexosaminidase (Hex), that causes progressive, rapid and fatal neurodegeneration in children by the age of 5. Moreover, this genetic disease is characterized by inflammatory features that are typical of all neurodegenerative diseases such as astrogliosis and microgliosis. Therefore, using a well characterized single gene disorder will allows us to demonstrate the anti-inflammatory effects of MSC-EVs on immune cells that are mainly involved in neuroinflammation before proceeding to in vivo experiments. Our objective was to evaluate the immunosuppressive properties of MSCs-EVs on astrocytes and, microglia in the feline in vitro SD model and compare to those normal cats. **Methods**. Brain tissue and whole blood from cats with SD and normal cats was used to isolate astrocytes and, microglia. Phenotypic characterization was performed via flow cytometry and immunofluorescence while 10^8 isolated from serum free conditions and characterized umbilical cord derived hMSCs - EVs were added to naïve and LPS-stimulated cells in order to assess their immunoregulatory effect. Cell culture supernatants were collected and analyzed for the production of pro-inflammatory cytokines, such as IL-6, through ELISA while mRNA expression of pro- inflammatory cytokine was assessed via reverse transcriptase quantitative PCR. **Results**. Isolated microglia and astrocytes from feline brain express cell specific markers, such as CD11b/c, CD18 and GFAP, accordingly. Also, our preliminary data indicate an immunomodulatory effect of hMSCs - EVs, through alteration in the mRNA expression of the pro - inflammatory cytokines in naïve and stimulated microglia and, astrocytes isolated from SD cats. Conclusions. These findings provide novel insights on how the hMSCs -EVs can be a promising tool against neuroinflammation in the SD feline in vitro model and their potential use in translational studies to fight diseases that are characterized by neurodegeneration, and neuroinflammation. **Acknowledgements**. Animal Health and Disease Research program.

974 New Technologies for CAR-T Characterization and Potency Testing

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Despite significant clinical advances in CAR-T cell therapies for cancer, the ability to precisely and reproducibly analyze the potency and identity of these drug products remains a challenge. As new CAR-T products and production methodologies are developed, it is becoming increasingly important to be able to rigorously evaluate the relative potencies of each batch. We have developed two MOA-based bioluminescent assays that overcome challenges in quantitative assessment of T cell therapies. Both assays rely on split NanoBiT luciferase technology. In the HiBiT Target Cell Killing Assay, incubation of CAR-T cells with target cells stably expressing HiBiT results in lysis of the target cells and release of HiBiT proteins. These HiBiT proteins bind with high affinity to LgBiT in the detection reagent and form functional NanoBiT luciferase, resulting in production of light in proportion to target cell lysis. Lumit Immunoassays are a simple and homogeneous method for measuring cytokine production from engineered T cells. Monoclonal antibodies are covalently labeled with LgBiT and a low-affinity tag, SmBiT, which have negligible association in solution. When the antibodies are brought together on a common analyte, such as a cytokine, SmBiT and LgBiT can compliment to form NanoBiT luciferase and produce light in proportion to the concentration of analyte in the sample. Lumit Immunoassays can be run directly on cells, removing media transfer, dilution, and wash steps that can be common sources of error. When autologous CAR-T products are being produced in parallel, it is essential to verify the identity of each product and ensure that it is administered to the correct patient. We demonstrate an easy workflow for STR analysis that can be used to document the chain of identity for CAR-T products.

975 The Unexpected Dual Impact of a Specific Transfection Reagent on the Biology of AAV

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AAV production - According to textbook, production of rAAV, without a helper virus, require nuclear extraction of packaging cells in order to release virions from cells. Nevertheless, many published protocols include a medium extraction step in addition to cellular extraction step. We have discovered that the use of the common transfection reagent - PEI specifically, to produce rAAV, resulted with substantial amount of virus released in the medium of packaging cells. **Methods:** HEK293T were transfected with packaging plasmids for production of AAV-GFP-DJ using PEI method at ratio of 1:2 or 1:3 (DNA to PEI) or using $CaPO_4$ method. Media was changed at 18 hrs post transfection; media from producing cells was collected by filtration (72 hrs post transfection) and AAV-GFP infectivity was estimated by serial dilutions (**Fig. 1A**). In addition, rAAV was extracted from cell pellet and viral genome (vg) copies were measured by qPCR (**Fig. 1B**). The amount of cellular rAAV is comparable with all transfection methods however a clear increase of virus in media collections is noticed with PEI transfections, especially when a ratio of 1:3 is used (x20 over $CaPO_4$). This phenomena was observed with all tested serotypes including, AAV1, 2, 5, 6, 8, 9 and many other recombinant capsids such as DJ, AAV-retro and Php.S. PEI induced rAAV secretion was not dependent on transfection itself and could be recapitulated by adding free PEI, without DNA, to medium of transfected cells (non PEI reagents), 24 hrs post transfection (data not shown). Interestingly, PEI induced rAAV secretion was not observed in suspension packaging cells but was regained upon adaptation of suspension cells to adherent growth in serum containing media (data not shown). **AAV infectivity** - Surprisingly, PEI has an additional inhibitory effect on rAAV infectivity however this effect was found to be serotype dependent and thus far is limited to heparan sulfate proteoglycan (HSPG) binding serotypes such as AAV2 and DJ but not AAV1 (**Fig.**

2). PEI inhibition was almost exclusively dependent on the addition of PEI to culture medium prior to virus inoculation. Incubation of heparin agarose with PEI prior to AAV2 binding and column purification had no impact on virus recovery from the column (data not shown). This preclude the possibility that PEI interfere directly with ligand binding of AAV2 but open up a compelling scenario where free PEI have an inhibitory effect on post ligand binding pathway of HSPG binding serotypes. This may hint that alternative internalization routes exist for AAV1 and AAV2 (and potentially other serotypes), and that the choice of route is directly linked with serotype specific ligand binding. Collectively, preliminary data suggest that linear polyethylenimine (**PEI**), commonly used as a transfection reagent for production of rAAV and other viral vectors have an unusual dual effect on AAV biology.

Fig. 1 Effect of PEI transfection on AAV-GFP secretion during production.

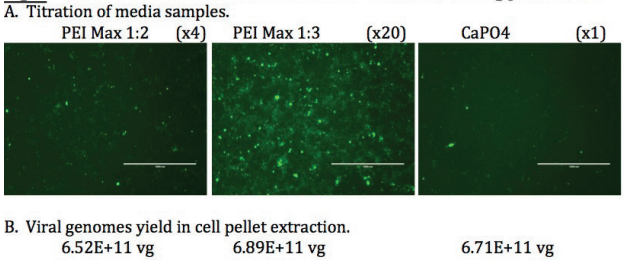
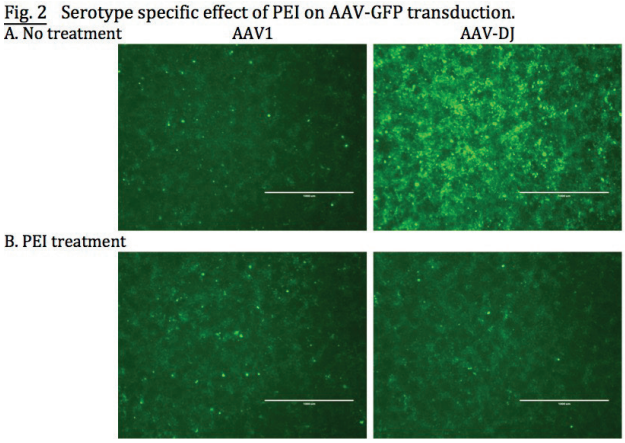


Fig. 2 Serotype specific effect of PEI on AAV-GFP transduction.



976 Development of an Intravitreal (IVT) Gene Therapy for Geographic Atrophy (GA) by Overexpressing Complement Factor I (CFI) to Inhibit the Complement Cascade

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Dry age-related macular degeneration (Dry AMD) with geographic atrophy (GA) is a highly prevalent disease, characterized by retinal pigment epithelium (RPE) and photoreceptor death leading to vision loss, which affects the quality of life in the aging population worldwide. Although the cause of Dry AMD is unknown, activation of components of the complement cascade have been associated with GA. More recently, data from clinical trials support that inhibition of the complement cascade can reduce GA lesion growth. Complement Factor I (CFI) is a rate-limiting enzyme in the complement cascade, naturally inhibiting the activity of proteins involved in complement overactivation. Therefore, continuous overexpression of CFI in the ocular tissue has the potential to inhibit the complement cascade in the eye, halt GA lesion growth and preserve vision in patients with Dry AMD. This can potentially be achieved by intravitreal (IVT)-delivery of adeno-associated viral (AAV) vectors engineered for broad retinal transduction and CFI expression. The ability to administer AAV vectors to patients via IVT delivery, a routine in-office procedure, is ideal for highly prevalent ocular disorders such as Dry AMD. In order to generate an AAV vector to treat patients with GA, we developed an improved codon-optimized CFI human cDNA (CFIco) sequence, which improved CFI expression over the wild-type sequence. This CFIco sequence was cloned into an AAV backbone under the transcriptional control of strong regulatory elements (named AAV-CFIco). Next, to evaluate the best AAV serotype for IVT delivery, AAV-CFIco was packaged into two of Adverum's proprietary highly retinotropic serotypes - AAV2.7m8 and AAV2.5T-LSV1 - which have shown to successfully transduce retinal cells following IVT delivery. After transduction of ARPE19 and rabbit retinal explants, both AAVs produced functional CFI protein in the culture media. To assess whether AAV2.7m8-CFIco and AAV2.5T-LSV1-CFIco could generate ocular CFI in a model system similar to humans, we performed a study using non-human primates (NHP). In this study, NHP subjects were administered bilateral IVT injections of AAV2.7m8-CFIco or AAV2.5T-LSV1-CFIco at either 3E10 vector genomes/eye (vg/eye) or 1E11 vg/eye (n=3/group), or vehicle (n=3/group). Vitreous humor (VH) was collected for CFI quantification on days 28, 62 and 88 post dose. Administration of AAV2.7m8-CFIco and AAV2.5T-LSV1-CFIco at both doses resulted in production of CFI. Mean peak CFI levels at the 3E10 vg/eye dose were 2464 ng/mL for AAV2.7m8-CFIco and 575 ng/mL for AAV2.5T-LSV1-CFIco. At the 1E11 vg/eye dose, mean peak CFI levels were 2313 ng/mL for AAV2.7m8-CFIco and 557 ng/mL for AAV2.5T-LSV1-CFIco. Overall, IVT administration of AAV2.7m8-CFIco and AAV2.5T-LSV1-CFIco in NHP subjects was well tolerated. No adverse systemic clinical signs were observed during the 3-month study. Animals had dose-dependent and self-resolving slight to mild ocular inflammation characterized by pigment and cells in the vitreous, and no abnormalities of the anterior segment or lens were found. In summary, the findings from the NHP study suggest IVT administration of either AAV2.7m8-CFIco or AAV2.5T-LSV1-CFIco at doses as low as 3E10 vg/eye (human equivalent dose of 6E10 vg/eye) may be capable of providing sustained therapeutic levels of CFI for the treatment of Dry AMD patients with GA. Importantly, the IVT route of administration allows for the routine in-office administration, something critical for this highly prevalent disease.

977 Gene Editing in the Lung Using Polymeric Vehicles

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The lungs remain a key organ of interest for the treatment of myriad diseases, such as cystic fibrosis, with gene therapy and gene editing therapeutics. Non-viral lipid- and polymer-based vehicles have shown promise for nucleic acid delivery *in vitro* and *in vivo*. However, the primary barrier for clinical translation remains delivery to target tissues *in vivo*. Local deposition throughout the airway can be achieved through inhalation and facilitates targeting of the epithelium. Inhaled mRNA delivery has been achieved through a variety of administration routes, including: intranasal, oropharyngeal, nebulization chamber, and intratracheal (IT) delivery. We have utilized IT administration to deliver both Cre mRNA (1) as well as CRISPR/Cas9-based gene editing agents using polymeric vehicles in the Ai14 reporter mouse model. We demonstrate that nucleic acid-based therapeutics can be effectively delivered *in vivo* using a class of polymeric vehicles consisting of members from a family of mildly cationic poly(amine-co-ester) (PACE) biodegradable polymers (2,3) that are designed for gene delivery and result in robust encapsulation of mRNA and sgRNA. Our initial studies focused on Cre mRNA delivery by PACE polyplexes via IT administration in the Ai14 mouse model. By flow cytometry, IT delivery of Cre mRNA results in ~10% tdTomato expression in bulk lung and ~30% expression in cells from bronchoalveolar lavage fluid (BALF) of treated animals. We further assessed which specific cell types in the lung were transfected: CD31+ endothelial cells, EpCAM+ epithelial cells, and CD45+ leukocytes. We observed ~20% of epithelial cells and leukocytes expressing tdTomato, with no detectable expression in endothelial cells. We next assessed the ability of PACE polyplexes to deliver SpCas9 mRNA and sgRNA to edit Ai14 mice. We measured tdTomato expression levels in the lung by flow cytometry 3 or 7 days after delivering one dose of gene editing PACE NPs. At day 3 post-treatment, the highest editing levels were observed in epithelial cells, though with less than 1% of cells exhibiting tdTomato expression by flow cytometry. Editing levels were significantly boosted when lungs were evaluated at day 7 post-dosing. In this case, we observed in ~0.5% of bulk lung cells, ~1.5% of leukocytes, and ~3% of epithelial cells, again by flow cytometry. We also imaged frozen lung sections and performed quantitative microscopy analysis of treated tissues. In this case, we quantified editing in small airways positive for tdTomato, by thresholding this signal based on the background signal from untreated control lung images. We then quantified the number of edited cells as a percentage of total cells in the airway. Using this analysis method, we observed ~60% editing in small airways on average. This number was similar among different lung samples analyzed from distinct animals. To obtain a broad sense of editing across a larger area closer to the size of a lobe, we tiled images of untreated control lungs as well as lungs from animals treated with IT-administered PACE NPs encapsulating Cas9 mRNA and sgRNA. We observe broad distribution/diffuse signal of tdTomato signal across the lung. On the whole, our findings demonstrate the promise of nucleic acid delivery mediated by inhalable polymeric carriers for the treatment of genetic lung disorders. **References** (1) Suberi A, et al. *bioRxiv*. 2022. (2) Grun M, et al. *Biomaterials*. 2021. (3) Kauffman A and Piotrowski-Daspit A, et al. *Biomacromolecules*. 2018.

978 Development of a Compact RNA Base Editor by Fusing ADAR with Engineered EcCas6e

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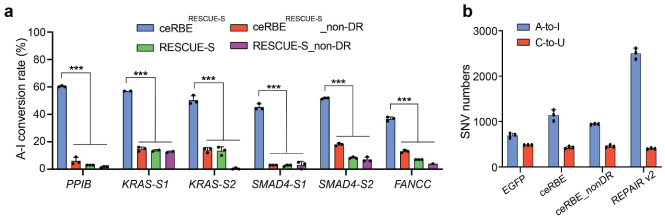
Background: Compared with genome editing, RNA editing is reversible and tunable without causing permanent changes in the genome, and it also has certain advantages in therapeutic applications. Among the numerous RNA base editing technologies, the base editor comprising a catalytically inactive RNA-targeting CRISPR-Cas13 (dCas13) protein and an RNA deaminase has great advantages in efficiency and editing types. This strategy has achieved efficient adenine-to-inosine (A-to-I) or cytidine-to-uridine (C-to-U) base editing *in vitro*. However, there are few *in vivo* studies due to its large size and inefficiency *in vivo*. Adeno-associated viruses (AAVs) are the most promising choice due to low immunogenicity and toxicity for *in vivo* delivery. However, the size of most base editors, consisting of dCas13 fused to an RNA deaminase, makes it difficult to package into a single AAV for efficient delivery. Here, we describe a smaller RNA base editor, ceRBE, with high efficiency *in vivo*. **Methods**: We selected ten small proteins, which belong to the CRISPR Class 1 system that recognize specific DR sequences and perform pre-crRNA processing to replace the dCas13 protein in the existing RNA base editor and explored base-editing efficiency *in vitro*. Then, the 199-amino acid EcCas6e protein was selected to optimize for toxicity and editing efficiency. Saturation mutation targeting H (His) 20 residue, a key site for pre-crRNA processing of EcCas6e protein, was performed to reduce toxicity. To improve efficiency, we optimized the editor with EcCas6e protein in three aspects: crRNA structure, subcellular localization, and spacer length. **Results**: By replacing the existing dCas13 protein with ten small proteins, we successfully achieved RNA base editing in HEK293T cells. The toxicity of the selected 199-amino acid EcCas6e protein with low off-target and high editing efficiency was reduced by saturation mutation targeting H (His) 20 residue, and editing efficiency was increased to approximately 1.3-1.8-fold by optimizing the crRNA structure, subcellular localization, and spacer length. Thereafter, the optimized **c**ompact and **e**fficient **R**NA **B**ase **E**ditor, consisting of EcCas6e^{H20L} protein, dual DR, one HIV Rev nuclear export sequence, and a 50 nt-length gRNA, is referred to **ceRBE**. Compared with the RESCUE-S editor with the same deaminase, the ceRBE editor shows a higher A-to-I and C-to-U editing ratio (A-to-I, 36.7~60.3%; C-to-U, 35.7~54.3%, Fig. 1a) and lower transcriptome off-target in HEK293T cells (Fig. 1b). To determine therapeutic potential, we packaged the editor into a single AAV and explored *in vivo* treatment of the *DMD*

Q1392X mutation in the humanized mouse model of DMD. The Q1392X (c.4174C>T) mutation results in the loss of dystrophin fibers in multiple muscle tissues. After 3 weeks of intramuscular injection of AAV, the proportion of dystrophin fibers was recovered from none to 68.1±9.4%, showing a good therapeutic effect. **Conclusions**: Overall, ceRBE, as a small and efficient RNA base editor, exhibits strong therapeutic potential for the genetic diseases.



979 Implications of AAV Affinity Column Reuse and Vector Stability on Product Quality Attributes

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While affinity chromatography is most preferred in the downstream processing of AAV vector products, the reuse of the AAV affinity columns is often avoided in the cGMP facility due to concerns over product quality inconsistency. Limiting the reuse of these expensive affinity columns is economically unfeasible. In this work, we examine the implications of AAV affinity column reuse and vector stability due to varying capsid design on the AAV9 product quality. An in-house pH gradient elution chromatography process with optimized clean-in-place (CIP) was developed to assess the reusability of the commercially available POROS CaptureSelect (PCS) AAVX and AAV9 affinity columns on AAV9 model vectors with varying viral protein (VP) ratios. Experiments with the drug substance AAV9 model vector feed indicated consistent vector elution behavior, independent of the vector VP ratio or column reuse cycle number. In contrast, the presence of feed impurities in the clarified lysate resulted in significantly different elution patterns during the affinity column reuse experiments. Analyzing the affinity eluates using a combination of analytical tools revealed that the product quality due to aggregation decreased over column reuse. Additionally, AAV9 serotype-specific PCS AAV9 column, with lower vector elution pH, resulted in higher aggregate content in the eluates as compared to the serotype-independent PCS AAVX column. Furthermore, the aggregation profiles also differed depending upon the model vector type with varying VP ratio. Interestingly, the vector aggregates also contained dsDNA and histone impurities. The levels of these impurities corroborated with the aggregation trends observed in these experiments. This indicates that the host cell impurities are likely carried over to the subsequent runs from column reuse due to inefficient CIP. These results suggest that the initial feed quality, affinity ligand type, elution pH, column CIP and vector stability can impact the reusability of AAV affinity columns and the product quality. This information can facilitate the rational design of a reusable AAV capture process with improved process economy and vector product quality.

980 SMART Transduction: A Framework to Control *Ex Vivo* Gene Transfer for Consistent, High Quality Cell Manufacturing

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Transducing cells with a viral vector is the critical step that transforms a patient's cells into a living drug by restoring or conferring new functions toward curative potential. Starting material variability and interdependent transduction parameters make process control and interpretation of outcomes challenging, which has far-reaching effects on safety, efficacy, durability, and cost. The current fixed manufacturing approaches using saturating viral vector dosages, extended ex vivo culture times, or both, prioritize generating large numbers of gene-modified cells without comprehensive consideration of cell quality. Here we present screening methodologies for achieving reproducible and targeted (SMART) transduction, a combined technological platform and analytical framework to efficiently design fit-for-purpose transduction. We leverage scalable high-efficiency transductions combined with statistical models to generate case-specific fundamental insights into optimal gene transfer and preservation of downstream critical quality attributes. SMART transduction uses a personalized data-driven approach for predictive capabilities of transduction outcomes, yielding more consistent high quality cell manufacturing processes that inherently minimize viral vector usage while remaining broadly applicable to many cell therapies such as CAR-T and CD34+ HSC gene therapy.

981 Technology Evaluation: Quicker Method for Lentiviral Vector Physical Titer Determination

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Measuring the HIV-1 p24 antigen is a long-established method for lentivrus quantitation. ELISAs can be laborious process that has numerous hands-on steps, and hours of incubation times. At Genezen, we were looking a way to decrease the time spent on physical titer determination. We evaluated ELLA instrument from Bio-Techne and VideoDrop as an alternative options to our regular p24 ELISA. ELLA uses a cartridge based system with capture antibodies and a then a fluorescent label that binds to your target, the ELLA based method streamlined and automated the immunoassay. The VideoDrop measures particles and gives a concentration of particles per mL. Initial method evaluation was performed by running Lentiviral vectors obtained from our internally developed process on the Genezen's regular ELISA assay and comparing that to the ELLA system's HIV-1 Gag protein kit. Correlation Coefficient was determined to be r=0.97 between the two methods. This is a strong positive linear correlation. Knowing that each kit could/would have different antibodies in their

assay, evaluating the trends between the samples seemed a more reasonable evaluation. Inter-analyst precision was performed. Out of 10 samples tested at 5 dilutions, there were 4 sample dilutions that had CV's between 20 and 26%. Other 46 sample dilutions were below 20% CV. Sample were also diluted across the standard curve and were found to give acceptable results out of 5 of the 6 dilutions for these samples. While the Viral Particle/mL compared between two methods, the ELLA had a Correlation Coefficient of r= 0.97 with the current Genezen ELISA method. ELLA's physical titer method was compared to the Genezen established ddPCR based Infectious titer method, and the Correlation Coefficient was 0.986. Same samples were tested on VideoDrop from Myriade. Lentivirus size ranges between 80-100nm. VideoDrop measures particles from 80 nm to 500 nm in size. In less than 1 minute the VideoDrop can analyze 7 uL of sample and produce a concentration values in Particles/mL. When compared Genezen ELISA data with he VideoDrop data, Correlation Coefficient was 0.94, which is considered a strong positive linear correlation. The VideoDrop based method could be used as a real-time in process check for Upstream and Downstream processing to reduce the time and costs.

982 Combining TRAIL-Secreting Induced Neural Stem Cell Therapy with a Novel Sensitizing Drug, TR-107 in Glioblastoma

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Glioblastoma (GBM) is an aggressive malignant adult brain tumor and is characterized by poor prognosis and survival outcome. Effective therapies are urgently needed as the infiltrative and heterogeneous natures of GBM render standard-of-care treatments futile—resulting in 90-100% tumor recurrence. Pro-apoptotic protein, tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is an ideal anti-cancer agent due to selectively killing cancer cells via death receptors 4 and 5 with negligible toxicity in normal cells. However, short half-life, insufficient drug accumulation at the tumor region, and development of drug resistance are critical challenges to overcome in TRAIL-based therapies against several cancers. Hence, our hypothesis is to target and activate crosstalk pathways in TRAIL-mediated extrinsic apoptosis for effective tumor suppression in GBM. Herein, we harnessed the tumor-homing capability of our human induced neural spheroidal stem cells (hiNeuroS) and engineered them to constitutively secrete and prolong TRAIL bioavailability at the tumor site. Furthermore, we combined hiNeuroS-TRAIL therapy with a novel drug, TR-107 to target crosstalk pathways for potential synergistic tumoricidal effects and to mitigate from establishing TRAIL resistance in GBM. *In vitro*, the combination therapy resulted in synergistic tumor killing effects with coefficient drug interaction values of **0.67** and **0.60** in LN229 and MS21 GBM cell lines, respectively. *In vivo*, combining a single intratumoral injection of hiNeuroS-TRAIL with intermittent intraperitoneal injections of TR-107 resulted in significant reduction of tumor growths and extended survival outcome compared to control and single agent treated groups in GBM tumor bearing mice.

983 Retroviral Replicating Vector-Mediated Prodrug-Activator Gene Therapy for Canine Cancers

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Retroviral replication vectors (RRVs) can selectively infect cancer cells and continue to spread efficiently within the tumor. Although not normally cytolytic, RRV can be engineered to directly kill cells by expression of a prodrug-activator enzyme that catalyzes intracellular production of a toxic metabolite from a nontoxic prodrug. RRV have been shown to achieve therapeutic benefit in a wide variety of human cancer models, but few studies have focused on veterinary malignancies. Here, we evaluated two different types of RRV, amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV) respectively, in experimental models of canine cancer.Both types of RRV were engineered to express GFP as a reporter gene, and replication was evaluated in 10 different canine cancer cell lines. Both AMLV- and GALV-based RRV achieved over 90% transduction by Day 30 in all these cell lines, but neither RRV replicated in canine primary normal fibroblasts and hepatocytes. Additionally, *in vivo* fluorescence imaging revealed that both types of RRV could spread efficiently through subcutaneous canine xenograft tumors (fibrosarcoma, hepatoma and lung cancer) in nude mice. Next, both types of RRV encoding the yeast cytosine deaminase prodrug-activator gene, which converts the prodrug 5-fluorocytosine to the active drug 5-fluorouracil, showed efficient killing of all 10 canine cancer cells in vitro in a prodrug dose-dependent manner. Furthermore, RRV-mediated prodrug-activator gene therapy achieved significant inhibition of subcutaneous fibrosarcoma tumor growth in nude mice.These data indicate that RRVs can be effective in the treatment of canine malignancies, and have potential applications in the veterinary field.

984 Characterization of the *Mottled-Blotch*y Mouse Model of Abnormal Copper Transport

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Menkes disease is a X-linked recessive severe neurogenetic disorder of copper metabolism characterized due to variants in the *Atp7a* copper transporter gene. A milder neurologic version of Menkes disease is modeled by the *mottled-blotchy* mouse (*mo-blo)* whose phenotype and genotype resemble human Occipital Horn Syndrome, first delineated nearly 30 years ago (*Nature Genetics*, 1994). The *blotchy* mouse phenotype is caused by an *Atp7a* "leaky" (non-canonical) splice junction variant that reduces but does not eliminate proper *Atp7a* splicing. Little is known about the quantity of correctly spliced Atp7a in *mo-blotchy*, a question relevant to viral gene therapy treatment approaches of these copper transport disorders. We sought to quantify the amount of properly spliced Atp7a transcript in *mo-blotchy* mice in order to better understand its phenotype and response to AAV9-

mediated gene replacement in combination with subcutaneous copper histidinate (CuHis). We documented *Atp7a* exon 11 skipping by RT-PCR analyses of *mo-blo* cDNA prepared from mouse brain, lung, liver, kidney and fibroblasts in comparison to normal tissues, as well as approximately 7% normally spliced *Atp7a* transcripts, based on quantitative RT-PCR relative to wild type. In conjunction with this molecular characterization, we evaluated dose responses to viral gene therapy in *mo-blo*. We identified one female pup with skewed X-inactivation The optimal dose regimen to date employs 7.6 x 10^{12} or 2.6 x 10^{13} vg/kg body weight of AAV9-*corsATP7A* in combination with 5mg of subcutaneous CuHis on P4, 5, and 6 which has resulted in 90% long term survival (n=10). We also identified one female pup with skewed X-inactivation leading to a severe phenotype and premature death. Cultured fibroblasts obtained from this mouse are under investigation for gene therapy mediated modulation of X-inactivation. The results from this study advance understanding of clinical and biochemical aspects of abnormal human copper transport and provide insight and direction for rational treatment approaches to Menkes disease and its milder variants.

985 OutSmart™ IL-2/15: A CAR T Cell-Produced Designed Cytokine with Tumor-Localized Immune Cell Activity

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Introduction: Chimeric antigen receptor (CAR) T cells are effective medicines against B cell malignancies, but CAR T efficacy for the treatment of solid tumors is impaired by insufficient CAR T proliferation and survival, and a lack of immune effector stimulation within the tumor microenvironment (TME). IL-2 is a potent T cell growth molecule that has been used as a protein therapeutic to encourage tumor-specific T cell proliferation. However, IL-2-related toxicity and T regulatory (Treg) cell stimulation limits clinical impact. We computationally designed OutSmart™ IL-2/15 lacking IL-2Rα binding to limit Treg activation and minimize toxicity, while retaining IL-2/15Rβγ binding interfaces and incorporating antigen-targeting to preferentially stimulate specific cell types such as CD8+ T cells and NK cells. We implemented OutSmart™ IL-2/15 as an integrated component of the genetic construct encoding the CAR, using novel promoters to regulate cytokine production at the tumor. Combining these design elements into a single vector module enables context-dependent, local delivery of the cytokine to the TME to enhance CAR T cell function, promote bystander immune cell function, and reduce the risk of toxicity. **Results:** Wild-type (WT) IL-2 was redesigned to replace IL-2Rα binding regions with short structured loops that increased protein stability and ablated interaction with IL-2Rα; the remodeled backbone results in a novel α-helix connectivity while retaining the WT IL-2Rβ and IL-2Rγ interfaces. 10,355 designs were generated, of which 38 were prioritized based on in silico analysis. 38/38 (100%) designs ablated IL-2Rα binding, and 33/38 (87%) designs retained IL-2/15Rβγ signaling. Designs were further optimized and prioritized based on biophysical characterization, phospho-STAT5 (pSTAT5) activation, and in vitro proliferation assays. Comparisons of pSTAT5 activation of CD8+ T cells, NK cells, and Tregs showed that WT IL-2 preferentially activated Tregs, whereas OutSmart™ IL-2/15 reduced Treg activity by 3-logs with minimal reduction in potency for CD8+ T cells and NK cells. T cells genetically modified with lentiviral vectors encoding WT IL-2 or OutSmart™ IL-2/15 showed that secretion was sufficient to drive T cell expansion in vitro. To enable context-dependent cytokine production from the CAR T cells, promoter/enhancer elements that respond to CD3ζ signaling were evaluated for low baseline activity and CAR-responsiveness following antigen engagement. We prioritized two "stim-on" promoters that demonstrated increased cytokine production following CAR activation by immobilized target antigen. OutSmart™ IL-2/15 production in response to CAR stimulation improved CAR T-mediated tumor killing and proliferation in repetitive tumor challenge studies. **Conclusions:** We demonstrate a genetic module that enhances CAR T cell function via tumor-localized IL-2/15 activity. OutSmart™ IL-2/15 preferentially expands anti-tumor immune effector cells, including NK cells and CD8+ T cells, while minimizing Treg activation via ablation of IL-2Rα binding. Our "stim-on" circuits leverage novel promoters to achieve local secretion of OutSmart™ IL-2/15 to enhance CAR T proliferation and survival, stimulate immune effector cells in the TME, and reduce potential side effects associated with systemic IL-2 exposure. Antigen targeting provides additional control by retaining the cytokine near the tumor and biasing activity to specific cell populations. More generally, OutSmart™ protein design pipelines and control technologies can be applied to create other designed cytokines, creating a powerful platform to achieve tumor-localized cytokine activity.

986 Spearheading Change from Scientist to Patients: The AI Impact on Cell & Gene

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It is projected that cell and gene therapies represent a $58 billion market opportunity over the next 5 years and hold the potential to treat millions of people with life-threatening diseases. While the cell and gene therapy space is a new frontier, the industry's focus should be on therapy development to scale and meet the substantial market for these novel therapeutics. More than just research and development, there is a growing need to focus on manufacturing process improvements that can overcome critical design issues and inefficiency that cost millions in wasted costs, elevated risk of adverse immune responses, and prohibitively high costs per dose. In recent years, AI technology has improved many industrial inefficiencies and can enable drastic design process improvements for cell and gene therapy pipelines. Digital tools are the next layer that can unlock operational challenges in production - through yield optimization with advanced analytics-based models, giving manufacturers proactive identification before any potential scaling issues occur.AI is the critical component and the prompt of this paradigm shift. The presentation will explore considerations for using proprietary AI models that analyze gene therapy constructs and optimize their operational process. First, the investment in an advanced AI technology stack built with large language models can be applied to biology problems. Model training for hyperparameter optimization and inference performance can enable a dramatically better experience, case studies showcasing 18x faster output, 5x cost-

effectiveness, and a 350% reduction in memory usage. The analysis can then be done as a more scalable and repeatable method. Form's state-of-the-art transformer neural network architecture for large language models identifies what's important when applied to DNA and protein analysis. Training this for specific biological tasks, such as construct replication efficiency leads to improvements in full capsids. AI is then taken to the next level, using coding and inference architecture for variant effect prediction and methylation to understand more deeply how changes can impact expression. Through training machine learning models based on specific, well-established DNA features, we understand multiple variables that impact truncation propensity, allowing visibility into secondary and tertiary structures that lead to truncation. While design for manufacturing via AI models has the potential for meaningful impact, practical application is of much more value. The presentation will feature a case study detailing our partnership with a publicly traded gene therapy company and how 30 days of AI analysis of AAV-based therapy predictively increased full reads by 18% and decreased truncations by 70% - increasing manufacturing yield, improving safety, and de-risking clinical development. Failures in replication and packaging of rAAV genomes into assembled capsids cause manufacturing efficiency/yield to plunge, accounting for millions of dollars in capital and months/years of manufacturing trial and error per pipeline candidate. In further detail, the presentation will break down. Evidence of how AI models can predict different truncation propensities in multiple indications across four gene therapy constructs with the same manufacturing process, as opposed to the reactive process of leading codon tools. Our never-before-performed technique of predicting and weighting a variety of 2o and 3o structures as part of construct design and to avoid increased mutation rates and replication machinery malfunction typically following secondary and tertiary DNA structures confirmations The biologics industry hasn't realized the potential of the newest advancements in machine learning to improve novel therapeutic manufacturing. Improvements like these can increase accessibility, and safety, and drastically decrease R+D timelines - to bring more doses to market and to patients in need.

987 Rational Designed Cationic Ionizable Lipids with Unique Hyperbranched Structures Exhibited Excellent *In Vivo* Nucleic Acid Delivery Efficiency

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Background: Lipid nanoparticles (LNPs) are currently being evaluated as a non-viral delivery system in clinical translation of *in vivo* genome editing technology. Transient expression of the gene editors of LNP/CRISPR Cas mRNA/gRNA cargo provides hit-and-run editing, which minimizes potential off-target risk to improve safety in clinical applications. Although excellent editing efficiencies have been reported in early-phase clinical trials for CRISPR-based therapeutics delivered by LNP, those were achieved by using very high doses (up to tens of mg) of RNA, which increases cost and tox risk. For the currently reported LNP technologies, it is estimated that less than 1~5% of the RNA payload of the endocytosed LNP escapes from endosomes into the cytosol. Therefore, there is room for improvement of the delivery efficiency by increasing the endosomolytic behavior of LNPs. Cationic ionizable lipid (CIL) is the most critical component in LNP formulation, which determines the cytosol delivery efficiency of the nucleic acid payload. CIL-mediated endosomal escape has been hypothesized to be a process associated with hexagonal II phase transition of lipid ion pairs, which might further trigger membrane fusion and disruption. However, this hypothesis has not been well-validated and rarely been applied in the discovery of new CILs. We therefore sought to develop novel CIL molecules with improved cytosol delivery efficiencies based on this hypothesis. **Methods:** Using a rational design and iterative screening strategy, a library of CIL molecules was synthesized and evaluated *in vitro* and *in vivo*. All CIL molecules synthesized have low molecule weights, with relatively simple yet hyperbranched structures. We hypothesized this unique molecular property could potentially facilitate hexagonal II phase transition. **Results:** After an extensive screening campaign in *in vitro* cell-based assays and *in vivo* studies in rodents, two lead candidates, VG-L61 and VG-L819, were identified exhibiting very high mRNA delivery efficiencies. The two novel CILs and a reference CIL, LP01, which was used in NTLA-2001 (the first *in vivo* CRISPR therapy tested in a clinical trial) were further evaluated *in vivo* using EPO mRNA as a reporter molecule. Following intravenous administration in mice, the serum EPO concentrations from VG-L61-based LNP and VG-L819-based LNP were 4.6 and 8.9 times that of LP01-based LNP, respectively. Interestingly, both VG-L61 and VG-L819 have hyperbranched structures with very short branching arms. Additionally, no increases of transaminases were observed in mice at 24 h post injection at doses up to 100 mg/kg of CILs. **Conclusions:** Using a rational designed strategy, novel CIL molecules were identified with significantly improved nucleic acid delivery efficiencies and good safety profiles. These results provided evidence for the hexagonal II phase transition theory, and also demonstrated that LNP delivery efficiency could be improved by further increasing the endosomolytic activities of the CILs. Experiments are ongoing to test these novel CILs for the *in vivo* delivery of genome editors.

988 Standardizing a Protocol for Streamlined Synthesis and Characterization of Lipid Nanoparticle to Enable Preclinical Research and Education

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Nanotechnology has made remarkable advances in clinical applications since the late 20th century. Due to the noticeable advantages in biocompatibility, drug delivery, and novel therapeutic and diagnostic applications, lipid nanoparticles (LNPs) have been used in gene therapy and vaccines. Recently, the clinical relevance was demonstrated by Pfizer and Moderna during the COVID-19 pandemic by making mRNA vaccines using LNPs. However, there are remaining challenges that impede preclinical work, such as expensive equipment and the lack of efficient delivery to non-hepatic tissue target. As more labs look to incorporate this technology, creating a standardized protocol at a relatively low cost would increase access and discovery. There are three methods that are commonly used to synthesize LNPs: pipette, vortex, and microfluidic mixing. LNP formulations generated *via* the microfluidics platform have been demonstrated to have enhanced properties for biomedical applications with increased loading efficiency

and proper size distribution. Microfluidic mixing is commonly performed using expensive equipment. For our purposes, we created a standardized protocol for microfluidic mixing using a syringe pump and an off-the-shelf microfluidic chip due to its comparatively low cost and high reproducibility. This protocol provides in-depth procedures on the synthesis of LNPs by microfluidic mixing and physical characterization using DLS (Zetasizer Nano ZS90, Malvern), nanoparticle tracking analysis (NanoSight NS300, Malvern), and evaluation of *in vitro* transfection efficiency and cytotoxicity. The protocol was created by recreating 3 well-known LNPs. Each LNP formulation contained an ionizable lipid: Cholesterol: DSPC: DMG-PEG. The ionizable lipids used were LP01, DLin-MC3-DMA, and SM102. DLin-MC3-DMA-LNP was made with a percentage ratio of 50:38.5:10:1.5, and we reported a size of 163 nm, a PDI of 0.140, a zeta potential of -5.94 mV, and an encapsulation efficiency of 99.4%. Similarly, LP01-LNP was made with a percentage ratio of 45:44:9:2, and we reported a size of 136 nm, a PDI of 0.10, a zeta potential of -12.6 mV, and an encapsulation efficiency of 94.7%. Lastly, SM102-LNP was made with a percentage ratio of 50:38.5:10:1.5, and we reported a size of 129 nm, a PDI of 0.10, a zeta potential of -36.77 mV, and an encapsulation efficiency of 94.9%. We observed successful transfection in HEK293 cells by each formulation mentioned above. In a 24-well plate, 75 ng-150 ng of GFP plasmid was added to the cells. A difference in expression was not observed for 100, 125, and 150 ng along with no observed cell death. Factors investigated to optimize manufacturing and size distribution was dialysis time, freeze-drying capabilities, reusability of microfluidic chips, and LNP stability after 30 days. We hereby report that our protocol will be used to fabricate LNPs to deliver mRNA to skeletal muscle and immune cells. To target immune cells, a new library of LNPs will be designed using the protocol to ensure the high probability of achieving an effective monodisperse sample.

989 Targeted Approach to Immunosuppression with AAV Gene Therapy: Nonclinical Support of Clinical Approaches

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Gene therapy using recombinant adeno-associated virus (rAAV) vectors has been successful in treating a wide range of human diseases. However, patients treated with rAAV can develop unwanted immune responses to the viral capsid, including B- & T-cell activation and transaminitis, which can lead to the loss of therapeutic transgene expression and formation of neutralizing antibodies, the latter hampering the potential for re-dosing. To combat the immune response to rAAV gene therapy and gene editing, corticosteroid usage has become more frequent over time in clinical trials, particularly with systemic AAV delivery. Given the broad mechanism of action of corticosteroids, adverse effects can be expected across a range of organ systems including hypertension, hyperglycemia, osteoporosis, and neuropsychiatric symptoms, such as insomnia and mood disturbance. Higher doses and longer courses of corticosteroids have been associated with an increased likelihood of developing more severe side effects. Because of these side effects, more targeted approaches are under investigation. In a 28-day study in cynomolgus monkeys, we investigated the effect of an immunosuppressive regimen on AAVHSC17-capsid specific nAb formation, immune activation, and transgene expression. The calcineurin inhibitor, tacrolimus (Prograf®), and the corticosteroid, dexamethasone, were administered prophylactically for immunosuppression, followed by a single intravenous (I.V.) dose of rAAVHSC17 expressing human phenylalanine hydroxylase (rAAVHSC17-PAH). The tacrolimus-and-dexamethasone immunosuppressive regimen used in this study reduced nAb formation against the rAAV capsid by 8-fold, reduced overall B- and T-cell activation, and increased transgene expression by 2-fold when compared to a control group administered rAAVHSC17-PAH without immunosuppression. The results of this study support the approach of a prophylactic combination of tacrolimus and dexamethasone in in conjunction with dosing in clinical trials of our investigational gene therapies. The pheEDIT and juMPStart trials are Phase 1 open-label, sequential, dose-escalation studies that will evaluate the safety and efficacy of a one-time I.V. administration investigational gene therapy vectors. The pheEDIT study is a geneediting vector and will be evaluated in adult participants with phenylketonuria (NCT05222178). The juMPStart study will evaluate the safety and efficacy of a one-time I.V. administration of HMI-203, an investigational gene therapy, in adult participants with Mucopolysaccharidosis Type II, also known as Hunter syndrome (NCT05238324) To decrease the potential for immune response to AAV gene therapy in these studies, a prophylactic immunosuppressive regimen of a corticosteroid in combination with tacrolimus are administered. The introduction of a T-cell inhibitor tacrolimus allows for a potential shortening of the duration of exposure to high dose corticosteroids and a more rapid taper. This combination therapy is anticipated to decrease the development of nAb, optimize transgene expression and reduce the adverse events related to corticosteroid therapy.

990 Improvement of Yield and Critical Quality Attributes through Process Development of a Novel Adeno-Associated Viral Vector Capsid (Anc80L65)

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Affinia Therapeutics is pioneering a new class of rationally designed gene therapies. We utilize our Affinia rationally designed Therapies (ART) platform to develop these therapies. One focus is developing novel capsids that enhance tissue targeting specificity, immunological profile, and manufacturability. One of the first capsids to come from this effort was Anc80L65 first identified by the Vandenberghe lab. In preclinical studies Anc80L65 has demonstrated rapid and broad gene expression in CNS compared to AAV9. We wanted to demonstrate that these rationally designed capsids can have similar manufacturing and stability profiles to natural serotypes. Multiple process development strategies were applied to improve total process yield and CQA's. Our platform upstream process

utilizes suspension HEK293 cells, so we started by testing different cell culture media, transfection reagents and transfection parameters utilizing Design of Experiment methodology. Through these efforts, we demonstrated over a log increase in yield at harvest in both shake flasks and bioreactors to >2e11 vg/mL. We then sought to develop a high yielding downstream purification method. We demonstrated that Anc80L65 can be purified with multiple commonly available affinity resins. We developed two polishing methods, with CsCl gradient we achieved >90% full capsids and for anion exchange chromatography we achieved >70% full capsids. We looked at other common critical quality attributes such as hcDNA, HCP, residual plasmid DNA and believe our process generates high quality vector. Stability is an important metric for AAV capsids, and we were able to show that Anc80L65 is stable at -80C for at least 12 months. Overall, we have demonstrated that Anc80L65 is a highly manufacturable capsid and gives us confidence our manufacturing platform will translate to newly discovered capsids.

991 Funding AAV Therapy for Sanfilippo Syndrome May Be More Cost Effective for Society Than Status Quo

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Rare diseases are frequently considered to be a secondary public health and research concern, yet the cumulative cost of all rare diseases have profound effects on society as a whole, and traumatic outcomes for families affected. Sanfilippo syndrome, for example, is an exceedingly rare genetic disease in the United States, has a small patient population and its research relies heavily on private donors. Sanfilippo syndrome causes profound neurological deficits, early death, and detrimental trauma to families for years, yet it's cumulative cost-burden in the United States has never been quantified. A multi-stage multiplicative comorbidity analysis was constructed using publicly available data and literature to simulate the predicted effects of Sanfilippo syndrome and its commensurate value to the public in USD based on the United States healthcare system's value of disability-adjusted-life-years (DALYs) presuming a 3% annual discount rate from 2020. The analysis demonstrated that, with the current standard of care for Sanfilippo syndrome, societal costs between 2020-2040 approximate to $1.34 billion, with the cost to individual families exceeding $8 million per child born with Sanfilippo syndrome. Societal costs approach $2.59 billion by 2100 despite a slowing birth rate in the United States, underscoring the cost of business-as-usual when funding individual rare diseases. Ultimately, this analysis supports the notion of increasing allocation of public research funds for rare diseases, such as Sanfilippo syndrome, not only from a humanitarian perspective, but also as a means of potentially reducing overall societal burden in a cost-effective manner. This analysis suggests that even allocating millions of dollars per year for Sanfilippo syndrome alone would potentially still be cost-effective compared to status quo, presuming novel treatments would be effective.

992 Assessment of Factors That Contribute to Inflammatory Toxicities During CAR-T Cell Therapy

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Inflammatory toxicities such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) are commonly associated with chimeric antigen receptor T cell (CAR-T cell) therapy. Although the mechanisms responsible for development of inflammatory toxicities are not completely understood, activation of bystander myeloid cells (MCA) and release of pro-inflammatory cytokines such as IL-6 and IL-1β following CAR-T cell therapy contribute to this. It is unclear how myeloid cells are activated during CAR-T cell therapy. GM-CSF produced by activated CAR-T cells has been suggested to activate myeloid cells; however, GM-CSF protein neutralization by antibodies or gene knock-out (KO) in T cells using CRISPR/Cas does not completely prevent MCA. This suggests that other T cell factors contribute to MCA. Fractionation studies of activated T cell supernatant identified a fraction of size range 100-300 kDa, which significantly activated myeloid cells compared to <100kDa and >300kDa fractions. Mass spectrometry analysis of 100-300kDa fraction identified several candidate proteins that may contribute to MCA. Protein function analysis and literature review identified a novel protein, GP1BA as one of the candidates. Neutralization of GP1BA in T cell supernatant using antibodies from 2 different sources significantly reduced MCA. However, GP1BA gene KO in T cells did not prevent MCA. Furthermore, recombinant GP1BA protein did not cause MCA. Interestingly, addition of anti-GP1BA antibodies in the supernatant from GP1BA KO T cells also prevented MCA. So far, these data suggest that T cell derived GP1BA does not contribute to MCA but GP1BA antibodies cross-react with an unidentified T cell-derived inflammatory factor, which contribute to MCA. Current studies are underway to identify inflammatory factor(s) that cross-react with GP1BA antibodies. Our long-term goals are to identify soluble factors released by CAR-T cells that contribute to inflammatory toxicities, and rationally engineer CAR-T cells that are safer and effective.

993 A Novel Fusogenix Proteolipid Vehicle-Based Platform for Efficient Delivery of Gene Therapy Products into the Eye

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Introduction: Common eye diseases leading to vision loss include, age-related macular degeneration, retinitis pigmentosa, diabetic retinopathy, stargardt disease, and glaucoma. Gene therapy offers a promising platform to reverse vision loss as well as treat the diseases which are untreatable today. Major barriers to the development of gene therapies for the eye include the inefficient delivery of nucleic

acids across different tissues and toxic side effects. The Fusogenix Proteo-Lipid Vehicle (PLV) technology overcomes these problems by employing the use of well tolerated lipids and the fusion-associated small transmembrane (FAST) protein, a small viral membrane protein that has evolved to induce cell-to-cell fusion and is non-immunogenic due to its small size.The goal of this study was to test whether our novel PLV can efficiently deliver nucleic acids *in vitro* in RPE cells and murine retina *in vivo*. Here, we investigated the use of our highly tolerable PLV formulations for eye tropism, by encapsulating different nucleic acids such as mRNA, pDNA, and siRNA. **Methods and Results:** PLVs encapsulating eGFP-mRNA and eGFP-DNA were first tested in *in vitro* models utilizing human RPE cell lines as well as isolated mouse retinal cells. PLVs showed high transfection efficiency with little to no cytotoxicity. Next, we tested the biodistribution of PLVs *in vivo,* using C57BL6 mice. PLVs encapsulating non-targeted luciferase-DNA (luc-DNA) were delivered via intravitreal injection. The *in vivo* and *ex vivo* imaging revealed expression of luc-DNA in the retinal layer and lens. Furthermore, immunofluorescence analysis revealed the co-localization of luciferase with different cell type-specific markers, specifically, tuj1 and rhodopsin, indicating a stable expression of DNA in retinal ganglion cells and photoreceptor cells. Furthermore, we tested PLVs encapsulating siRNA for functional protein KD following intravitreal administration, and observed ~70% knockdown. Finally, we tested the off-target effects of PLVs compared to commercial MC3-LNP formulations following intravitreal administration of mRNA-Luc. A strong luciferase expression was detected in the liver of MC3-LNPs, whereas PLVs did not show any luciferase signal. **Conclusion:** Our results indicate that Fusogenix PLVs can be used to effectively and safely deliver mRNA, pDNA, and siRNA *in vitro* and *in vivo*, with broad biodistribution in the murine eye, and unlike conventional LNPs, has no off-target effects. We demonstrate that Fusogenix-PLV offers a promising gene delivery platform for eye therapeutics.

994 Comprehensive Analysis of the AAV9 Galactose-Binding Pocket and Its Implications for AAV Vector Biodistribution

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The natural tropism of adeno-associated viruses (AAVs) for the liver represents a major impediment to the expansion of AAV gene therapy into clinical indications affecting non-liver peripheral organs such as the heart. Recent deaths and adverse events associated with liver toxicity in high-dose AAV gene therapy trials targeting peripheral organs exemplify this problem. Thus, there is a need for novel AAV vectors that are detargeted from the liver but retain the ability to transduce peripheral organs. The primary cellular receptor for AAV9 is galactose, the penultimate glycan residue of the N- and O-linked glycan chains that adorn the surface of all cell types. Our group initially described the galactose-binding pocket on the surface of AAV9 as a cluster of five amino acids, N271, D272, Y446, N470, and W503. Previous work from our lab showed that alanine substitutions at these positions ablate *in vitro* transduction. Moreover, our unpublished data show that the substitution W503A reduces *in vivo* transduction to 0.02x expression in mouse liver and 0.15x expression in mouse heart compared to AAV9. Data from other groups suggest that amino acid substitutions other than alanine can confer similar levels of liver detargeting while preserving relevant levels of peripheral transduction. Thus, we performed multiple screening rounds of an AAV library containing variations at the AAV9 galactose binding site and identified a vector with a 2.7x enhancement in cardiac transduction with a concomitant 0.43x decrease in liver transduction in mice. The generation of liver-detargeted AAV vectors that exhibit enhanced cardiac transduction represents important progress in improving vector specificity and safety.

995 The Impact of the *In Vitro* Diagnostic Medical Devices Regulation (EU) 2017/746 on Gene Therapy Medicinal Product Developers

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The EU In Vitro Diagnostic Medical Devices Regulation (IVDR) (2017/746) was developed to specify the safety, integrity, and quality requirements for any in vitro diagnostic (IVD) manufactured or used in the EU. The regulation carries few exemptions, including none for early-phase clinical studies, orphan products, or gene therapy medicinal products (GTMPs). It came into effect on 25 May 2017 with a 5-year transition period. With the end of the IVDR transition period on 26 May 2022 and the mandatory submission of initial clinical studies under the EU Clinical Trial Regulation (CTR) (536/2014) after 31 January 2023, the IVDR poses major potential hurdles to the development of many GTMPs in the EU. For GTMPs, anti-drug antibody (ADA) assays are commonly used to determine patient eligibility for clinical trials by characterizing patient immunogenicity to a viral vector capsid and/or the expressed transgene. These assays are considered in vitro diagnostics (IVDs) or companion diagnostics (CDx) and under the IVDR are subject to the full scope of the regulation. This means that the submission and acceptance of clinical performance study plans are needed to initiate participant enrollment in the clinical trial in addition to the approval of the clinical trial application (CTA). In the case of ADA assays for orphan products, there are limited published data or commercial assays to justify an alternative to the development of a CDx, and the ability to assess clinical performance in the patient population is significantly challenged due to limited access to low prevalence patient populations. In light of this transition, Forge will discuss some important points for GTMP developers to consider before initiating a clinical trial in the EU that uses a CDx or IVD. These include opportunities for scientific advice during the development of your performance study and CTA strategy, collection of robust analytical performance data during assay development, considerations for the design of a clinical performance study (including using the trial itself), and advanced planning for late-stage development of the CDx, like potential partnership opportunities. This presentation will also include a comparison to IVD and CDx regulations in the US. The IVDR adds a layer of complexity to the conduct of GTMP clinical trials in the EU and necessitates that sponsors give it ample consideration early in the development of their clinical trial strategy.

997 Validation of an Analytical Method for Lentivirus Physical Concentration Using Videodrop (ILM) Following ICH Guidelines

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The EMA guidelines for quality control of gene therapy medicinal products recommend controlling the particle number and size of viral vectrs drug substance (DS), drug product (DP) and critical intermediates in the viral vector process. Videodrop is an innovative system based on Interferometric Light Microscopy (ILM) which measures nanoparticle concentration and size quickly and easily. it cab be included in the quality control plan. For this purpose, analytical methods must be validated following ICH Q2 guidelines. This study proposes evaluating the linearity and precision of Videodrop by analyzing lentivirus samples from various stages of bioproduction. The validation study includes two different bioprocesses and more than 200 measurements. The results of this validation study show a very good precision with CV<15% and excellent linearity with R^2 >0,97. Results are favorable and hold promise for GMP method validation for gene therapy products with Videodrop. Moreover, the very limited number of parameters and ease of use allow a very high reproducibility allowing easy QC transfer.

998 Mouse CD123-CAR T Cells Elicit Robust Anti-AML Activity without HSC Toxicity in Syngeneic Models

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The use of chimeric antigen receptor (CAR) T cells specific for acute myeloid leukemia has been hampered by limited expansion and efficacy in clinical trials, despite robust antitumor activity in preclinical models. Understanding the interactions between CAR T cells and the highly immunosuppressive acute myeloid leukemia (AML) tumor microenvironment (TME) is essential to improving this therapy. To date, there are no syngeneic AML models using AML-CARs that mimic the aggressive TME. Here, we established the first mouse CD123 (mCD123)-CAR T cell immunocompetent system. We designed 4 second generation mCD123-CARs with single chain variable fragments (scFvs) derived from biopanning of a mouse phage display library on recombinant mCD123. Only one of the CARs was successfully expressed on activated mouse T cells from splenocytes as detected by F(ab')2 (55.8±11.5% N=7,) or recombinant mCD123 (59.0±12.1% N=6). To measure antigen specificity, we expressed full-length mCD123 on 2 mouse leukemia cell lines: C1498, a spontaneous myelomonocytic AML cell line, and an Arf-null, BCR-ABL1 pre-B-ALL (B-ALL^{123+}). We observed significantly increased cytokine secretion and cytolytic activity in mCD123-CAR T cells when cocultured in the presence of CD123+ targets in comparison to controls (N=6, p<0.01). Antitumor activity was maintained in repeat stimulation assays for up to 5 stimulations. *In vivo,* mCD123-CAR T cells had significant anti-leukemia activity against B-ALL^{123+} resulting in a prolonged survival advantage in comparison to controls (N=5 / group, p<0.001). In addition, we analyzed a panel of virally induced NUP98::KDM5A AMLs generated by transduction of mouse hematopoietic progenitor cells and determined all samples naturally expressed mCD123 antigen at different densities (range 38-77%; N=4). We evaluated the anti-leukemic activity of mCD123-CAR T cells in vivo. Our data show significantly reduced leukemia burden (p<0.001) and extended survival (p<0.01) in mice treated with 5 x10^6 mCD123-CAR T cells compared to control CAR T cells. We detected mCD123-CAR T cells in treated mice using RNAscope probes. To measure toxicity, we injected non-tumor bearing mice with 5x10^6 luciferase modified mCD123-CAR T cells. We monitored expansion for 10 days and analyzed bone marrow and spleen on day 10 using flow cytometry and CFU assays. We noted no differences in toxicity profile of mCD123-CAR compared to controls. Next, we delineated the TME in our models using flow cytometry and single cell transcriptomic data from bone marrow of mice injected with B-ALL^{123+} +/- mCD123-CAR T cells ~7 days post T cell injection. Our findings reveal that the TME of our immune competent leukemia CAR T cell therapy models recapitulate human disease, showing an increase in dying tumor cells within the bone marrow of mice in the treated group, with a number of other populations (e.g. dendritic cells, regulatory T cells) approximating the tumor-only microenvironment profile. We have developed the first functional mCD123-CAR T cells targeting mouse AML and ALL and have shown their activity in immunocompetent models, with potent anti-leukemic activity *in vitro* and *in vivo* with minimal toxicity. Our model will provide insight into the impact of the AML TME on CAR T cell functionality and inform future strategies to improve CAR T cells in the immunosuppressive TME.

999 Variant-Aware Off-Target Analysis of Therapeutic CRISPR Gene Editing Targets

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CRISPR gene editing therapy holds tremendous promise for the treatment of inherited genetic diseases. Many genetic disorders display population-specific differences in prevalence. Sickle Cell Disease (SCD) and *APOL1*-mediated kidney disease represent two examples of such genetic diseases with enrichment in individuals of African ancestry. The FDA has recently emphasized the importance of genetic analysis to understand the effects of human variation on editing activity across intended to treat populations in its draft guidance for gene editing therapy development. In this study, we applied oligonucleotide enrichment and sequencing (ONE-seq) in a variant-aware manner to identify candidate off-target sites with variable editing potential and allele frequency across major human populations. We assessed two gene editing targets that are currently being evaluated in human subjects in early-stage clinical trials:

one in *BCL11A*, a regulator of the developmental switch from gamma- to beta-globin for treatment of SCD and β-thalassemia, and one in *PCSK9*, a regulator of LDL receptor and plasma cholesterol levels for treatment of hypercholesterolemia. Phased variants derived from the 1000 Genomes and Human Genome Diversity Project data sets (3,433 genomes) were evaluated for their quantitative effect on Cas9 gene editing in vitro. ONE-seq analysis identified a large number of candidate off-target sites in which variants either increased or decreased in vitro editing frequency substantially compared to the reference sequence. Many of these variant-specific off-targets had remarkable effect sizes, often over 1000-fold in some cases. The nominated sites were subsequently analyzed to identify those where increased editing at variant sites could potentially alter the function or expression of associated genes resulting in significant biological impact. Together, these results underscore the critical importance of including genetic diversity across human populations as a consideration in evaluating potential off-target gene edits.

1000 Dissecting the Epigenetic Regulation of Hemoglobin Expression to Unravel Novel Curative Options for β-Hemoglobinopathies

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β-hemoglobinopathies are severe genetic diseases caused by mutations affecting the production of the β-globin chain. The clinical severity of these pathologies is mitigated by the co-inheritance of mutations that reactivate *γ-globin*(*HBG*) genes expression and result in elevated production of fetal hemoglobin (HbF) in adults, a condition named hereditary persistence of HbF (HPFH). Multiple strategies to reactivate *HBG* were described, such as the introduction of HPFH mutations in their promoters that result in *de novo* binding sites for transcriptional activators. HbF reactivation in adult cells can also result from down-regulation of *BCL11A*, a gene encoding a major *HBG* transcriptional repressor.Allogenic hematopoietic stem/progenitor cell (HSPC) transplantation is the only definitive therapy for patients with β-hemoglobinopathies. In the absence of a compatible donor, transplantation of corrected autologous HSPCs represents an attractive therapeutic option. Lentiviral vectors (LV) or CRISPR/Cas9 have been exploited to restore β-globin expression. However, genotoxicity due to the semi-random LV integration in the host genome or to unwanted activity of CRISPR/Cas9, raises safety concerns for clinical applications. Therefore, novel and safer therapeutic strategies for β-hemoglobinopathies need to be developed. We investigated the epigenetic regulation of *HBG* and *BCL11A* to develop novel therapies via targeted epigenome editing with the goal of modulating gene expression without modifying the underlying DNA sequence. To this aim, we analyzed epigenetic marks at two key *cis*-regulatory regions, the *HBG* promoters and the *BCL11A*enhancers, in patients' HSPC-derived erythroid cells. Bisulfite sequencing revealed high DNA methylation of the *HBG* promoters in erythroid cells expressing mainly adult hemoglobin. On the contrary, introduction of HPFH mutations that recruit the KLF1 transcriptional activator at the *HBG* promoters led to reduced DNA methylation and high HbF production. These results indicate a role for DNA methylation in *HBG* repression which, however, can be reverted in adult erythroid cells to induce HbF expression. The *BCL11A* enhancers were modestly methylated in adult erythroid cells that express *BCL11A*. CRISPR/Cas9-mediated inactivation of activator binding sites mapping to these enhancers led to increased DNA methylation associated with *BCL11A* downregulation and HbF reactivation. These findings suggest that DNA methylation can negatively impact the activity of *BCL11A* enhancers and can be modulated to reactivate HbF.Furthermore, we used ChIP-seq to profile the presence of defined histone marks at the two *cis*-regulatory regions analyzed. Deposition of active histone modifications correlated well with the activity of the *HBG* promoters and *BCL11A* enhancers in primary adult erythroblasts. Indeed, in fetal primary erythroblasts expressing γ-globin, *HBG*promoters showed high levels of histone 3 lysine 27 acetylation (H3K27ac) and lysine 4 trimethylation (H3K4me3), typically associated with active transcription, while these modifications were absent in adult primary erythroblasts that do not express γ-globin. Current experiments aim to exploit designer epigenome modifiers to manipulate the epigenetic architecture of the *HBG* promoters and the *BCL11A* enhancers to achieve persistent HbF expression in patients' HSPC-derived erythroid cells. We anticipate that the epigenome editing strategy pursued here overcomes genotoxicity risks and offers a potentially safer approach for the future treatment of β-hemoglobinopathies.

1001 Intra-Thalamic Targeting for Widespread Brain Biodistribution of AAV-Based Gene Therapies

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Harnessing the full power of gene therapy (GTx) heavily relies on targeting essential tissues and cellular populations with specificity and sensitivity. This is particularly complex in the central nervous system (CNS) as many CNS disorders require widespread biodistribution over multiple brain regions for therapeutic efficacy. While intra cerebrospinal fluid administration (Intracerebroventricular, Intracisterna magna) is feasible, and blood-brain barrier crossing capsids for systemic injection are in development, these methods pose a greater risk of unwanted peripheral tissue transduction. Another approach is to take advantage of the native neuronal projections to and from major brain network hubs and utilize them to deliver AAV GTx's to anterograde and retrograde connected brain regions to reduce the risk of peripheral tissue transduction. To this end, we have evaluated several intra-thalamic injection coordinates with the goal of wide-spread cortical and hippocampal transduction using AAV serotype 9 expressing enhanced green fluorescent protein (AAV9.eGFP) constructs. We first utilized anterograde and retrograde tracers (dextran AF-647 and CTB-AF555, respectively) to determine coordinates resulting in cortical and hippocampal transduction using traditional immunofluorescence or whole brain tissue clearing, followed by light sheet microscopy imaging (LifeCanvas Technologies) and analysis. We found distribution of the tracers along the antero-retrograde thalamic projections throughout the anteroventral regions of cortex with each of our tested stereotaxic

coordinates. Additionally, AAV9.eGFP transduction using similar coordinates closely recapitulated the biodistribution observed using the tracers. Furthermore, we tested whether single stranded (ssAAV) and/or self-complimentary AAV (scAAV) genomes impact the biodistribution of eGFP expression using the optimized intra-thalamic coordinates. We found that scAAV had superior biodistribution to the cortex and hippocampus when compared with ssAAV. These findings have broad implications for CNS GTx therapeutics and are informative for CNS diseases that require broad brain transduction.

1002 An Innovative Platform Approach for the Development of *Ex-Vivo* Gene Therapies for the Treatment of Lysosomal Storage Diseases with Skeletal Involvement

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Lysosomal storage diseases (LSDs) are characterized by the accumulation of undigested macromolecules causing severe multi-organ damage, which the currently approved therapies fail to cure. Previous data obtained by our Institute proved the safety and efficacy of Hematopoietic Stem and Progenitor Cell-Gene Therapy (HSPC-GT) for the treatment of other LSDs. Building on this experience and the common pathological mechanisms of LSDs, we are proposing HSPC-GT to cure a group of rare/ultra-rare LSDs with skeletal involvement (Mucopolysaccharidosis IVA, Mucopolysaccharidosis IVB, Alpha-Mannosidosis) by using a standardized process in the framework of an innovative platform approach. We optimized Chemistry, Manufacturing and Controls, Non-clinical and Clinical development plans to generate a platform dataset complemented by specific disease data to generate a single combined Clinical Trial Application for clinical testing, moving from the "1-to-1 sequential" drug development to the "simultaneous and parallel" development approach. To this aim, we generated 3rd-generation lentiviral vectors encoding for each specific enzyme (LV-GALNS, -GLB1, and -MAN2B1) to transduce human HSPCs. Transduced cells showed proper clonogenic and proliferative capacity and significantly overexpressed the functional enzyme. Preliminary data also indicated that the enzymatic activity was restored in patients' derived fibroblasts exposed to the conditioned medium from LV-GALNS and MAN2B1 transduced human HSPCs-derived myeloid cells. Importantly, osteoclasts derived from LV-GALNS and GLB1 transduced cells abundantly secreted the different lysosomal enzymes, possibly serving as a resident enzyme source for skeletal cross-correction. Moreover, LV-GALNS transduced HSPCs engrafted and differentiated in the hematopoietic organs when transplanted in NSG mice. Altogether, our pre-clinical data support the HSPC-GT development for the platform LSD treatment.

1004 Anc80 Delivery of the RPGRIP Gene Stabilizes Functional Vision Decline in a Genetic Mouse Model

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Homozygous and compound heterozygous loss-of-function mutations in *RPGRIP1* are associated with several rare retinal dystrophies, most commonly with the diagnosis of Leber congenital amaurosis type 6 (LCA6) but also associated with cone-rod dystrophy 13 (CORD13) and forms of juvenile-onset retinitis pigmentosa (jvRP). LCA6 presents in early childhood with the most severe forms appearing as early as birth. Odylia Therapeutics is developing a gene therapy to treat vision loss caused by *RPGRIP1* mutations. This gene therapy will utilize the Anc80 AAV capsid to deliver a functional copy of the *RPGRIP1* gene to photoreceptors. To-date, strong proof-of-concept data has been generated in a mouse model of LCA6 and preliminary non-human primate safety data supports vector tolerability at the doses tested. Odylia is currently in the final stages of preclinical testing while planning for IND submission. Despite that the estimated prevalence of LCA6 in the United States is approximately 450-1,350 individuals, Odylia champions this and other programs by partnering with a diverse network of collaborators to ensure that promising therapeutics aren't left behind.

1005 Synthetic DNA and Targeted Non-Viral Delivery Systems for Genetic Correction of Duchenne's Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe monogenic disease caused by mutations in the gene encoding dystrophin. While several trials are on-going, including AAV-mediated delivery of a truncated micro-dystrophin, no clinically effective therapy is available to date. The emergence of CRISPR-Cas and associated editing technologies provide an attractive therapeutic option for the treatment of DMD, however delivery to muscle remains a challenge. 4basebio has developed a proprietary, non-viral nanoparticle that targets muscle cells with high specificity and low immunogenicity, allowing for repeat dosing. The Hermes™ particle is payload agnostic and has been used to deliver DNA, mRNA and proteins to muscle cells both *in vitro* and *in vivo*. In addition, we have a scalable enzymatic synthesis process for the production of linear DNA constructs via our Trueprime amplification technology. The oeDNA produced is devoid of any bacterial backbone and the manufacturing process circumvents cumbersome fermentation processes required for plasmid DNA, is size and sequence independent, and ideally suited for DNA repair templates used in gene editing. In this study, we utilised the muscle targeting particle to deliver Cas9 protein

with an sgRNA targeting the DMD gene in C2C12 myoblasts and myotubes. Particles had highly favourable biophysical characteristics, stability, encapsulation of payload, and achieved high KO efficiency in relevant cell models. To investigate a gene-integration approach using our plasmid-free synthetic DNA technology, we designed an oeDNA™ template encoding the cDNA sequence of the *Dystrophin* gene with flanking homology arms. The co-delivery of template DNA along with the RNP complex encapsulated in our Hermes™ nanoparticle resulted in a successful integration of the oeDNA template, and enhanced protein expression *in* vitro. Mouse studies confirming these findings are ongoing. The present study provides proof-of-concept for the use of Hermes™ particles to deliver RNP + donor templates to reframe or fully replace a dysfunctional gene, offering a promising approach for DMD therapy.

1007 Allogeneic Class I MHC Gene Therapy Induces Tumor Rejection in a Murine Model of Hepatocellular Carcinoma

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The incidence of hepatocellular carcinoma (HCC) in the United States has almost tripled in the last 20 years. Recently, immune checkpoint inhibitors have improved outcomes for treatment of HCC. However, the efficacy of immune checkpoint blockade can be limited by an inherently immunosuppressive environment within the liver, as well as tumor cell downregulation of class I MHC to evade cytotoxic T cell detection. Another promising treatment approach for HCC is gene therapy, which can either target a tumor specific gene or induce expression of immune modifiers.In order to overcome immune ignorance of HCC tumors, we formulated a gene therapy mediated approach to induce expression of Class I MHC on tumor cells. We hypothesized that **gene therapy induced expression of allogeneic class I MHC on cancer cells** would lead to an immune response towards the tumor, in a manner similar to graft rejection. We therefore engineered both an integrating lentivirus (LV) and a non-integrating AAV serotype 8 (AAV8) platform, each encoding either matched (H-2Kb) or allogeneic H-2K (H-2Kk and H-2Kd) murine class I MHC alleles along with a luciferase reporter. We first validated that our viral vectors induced detectable expression of foreign MHC following LV infection in HCC cell lines *in vitro.* We then established a murine syngeneic model of HCC in C57Bl/6 mice and treated tumor-bearing mice with three intratumoral doses of viral vector. We assessed therapeutic efficacy by comparing tumor growth of MHC-match and MHC-mismatch treated groups to luciferase-only vector and saline control conditions. We were able to detect efficacious gene delivery and *in vivo* expression by luciferase activity in treated murine tumors. Both gene therapy approaches were well tolerated in mice, with no observable detriments in mouse body weight or behavior. Tumor rejection and improved long-term survival was observed in 80% of the H-2Kk LV treated mice and 43% of H-2Kd LV treated mice. Substantial difference in tumor growth was observed in both H-2Kk AAV8 (62.9% mean tumor inhibition) and H-2Kd AAV8 (84.8% mean tumor inhibition) treated mice after three doses. We also performed immunoprofiling of tumor infiltrating lymphocytes at endpoint in remaining tumors and observed tumor infiltrating CD8 T cells were increased in H-2Kk AAV8-treated mice. Although gene therapy mediated expression of self MHC in the tumors exhibited a modest effect on tumor growth in LV and AAV8 treated mice, the results were more profound when inducing allogeneic MHC expression.These results show for the first time that gene therapy induced expression of allogeneic class I MHC can stimulate tumor rejection in a murine model of hepatocellular carcinoma. Further work will improve upon viral targeting to HCC to allow for intravenous or peritoneal delivery, as well as combination therapy with immune checkpoint inhibitors.

1008 Genetic Medicine Using a PLV Approach Reduces Frailty and Improves Healthspan

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Physical frailty and sarcopenia affect half of all elderly individuals and can lead to poor quality of life disability, and death. It is also linked to cardiovascular diseases, respiratory disease, and cancer in middle age, with the underlying mechanisms still being investigated. Senescence, a cell fate characterized by quiescence and secretion of pro-inflammatory molecules, is a hallmark of aging that is implicated in a variety of age-related pathologies. Eliminating senescent cells (SCs) in vivo using transgenic mice have demonstrated significant improvements in healthspan and amelioration of age-related degeneration, including physical frailty and sarcopenia. However, since this requires genetic engineering of the organism from the embryo, there is broad active development on senolytic drugs. This new class of therapeutics are designed to mimic the effect and increase health span. The first generation of senolytics have been repurposed drugs with off-target effects and tolerability limitations. Thus, next generation interventions require better specificity and tolerability. Herein we discuss senescence-dependent and senescence-independent clinically viable gene therapies to combat frailty and improve muscle function. The first consisting of a suicide gene under a senescent cell promoter delivered in vivo with Proteo-Lipid Vehicles (PLVs). These PLVs employ fusion-associated small transmembrane (FAST) proteins that can efficiently transduce a wide range of cells in vivo. Selective ablation of target cells is then achieved through the expression of a potent pro-apoptotic transgene driven by a specific senescence-associated promoter such as p16Ink4A or p53. The second consisting of expressing the myostatin and activin antagonist, Follistatin, under the control of a promoter specific to the liver where it is majorly expressed for muscle growth. We examined the effect of senolysis by our FAST-PLV senolytic therapy on healthspan utilizing a battery of clinically translatable metrics for physical function and frailty. Naturally Aged C57/B6 mice 24-26 months of age were treated monthly with senolytic PLVs encoding a suicide gene driven by senescence-associated promoters p16 or p53. Clinical frailty measured by deficit accumulation was assessed using 31 observational metrics, physical function was assessed with a battery of tests (grip strength, rotarod, treadmill, and open field), and heart function was assessed using ECG. Aged mice treated with senolytic

PLVs showed significantly reduced senescent cell burden body wide. Treatment with senolytic PLVs increased median post-treatment survival by 160%, lowered clinical frailty, and improved physical and heart function. Additionally, spontaneous tumor burden in these mice was reduced by 60%. Our data shows that senolytic FAST-PLVs improved healthspan and reduced frailty in naturally aged mice. We separately measured the effect on muscle growth by a FAST-PLV Follistatin therapy. Up to 9 months after one treatment, muscle weight and fiber size were significantly increased resulting in increased body weight and functional grip strength. In summary, our approaches represent a first-in-class therapeutic that targets cells based on transcriptional activity, rather than surface markers or metabolism, and represents a viable strategy for treating age-related diseases such as physical frailty and sarcopenia.

1010 Development of Rational Formulation for the Delivery of AAV Viral Vector for Treatment of Heart Disease

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Adeno-associated virus (AAV) has emerged as a leading platform for gene delivery for treating genetic heart diseases. However, due to challenges associated with manufacturing scale up and long term storage, AAV can be prone to degradative, aggregative, and adsorptive loss which can negatively impact the purity, potency, and quality of the final drug product. Additionally, for the treatment of heart disease in particular, depending on route of administration, conventional drug product formulation excipients, such as phosphate and acetate, can have negative effects on heart function, which limit the options for excipient selection. Using rational design, Tenaya Therapeutics, Inc. has developed a robust formulation for AAV that is stable under different storage temperatures and stress conditions. Additionally, the formulation is photostable and supports in-use conditions including multiple freeze-thaw cycles, and is compatible with devices for both intravenous and local delivery via cardiac catheters, greatly enhances clinicians' ability to bring and deliver AAV viral vector drug products to patients suffering from genetic heart diseases.

1011 Development of GyroLab *In Vitro* Immunoassay to Detect Pre Existing Antibodies Against AAV

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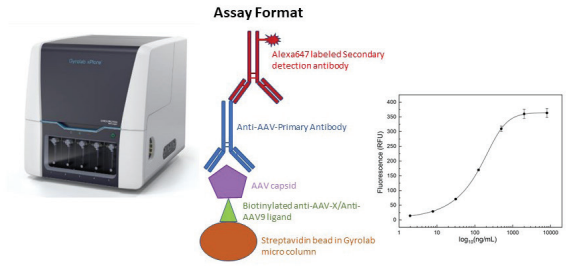
Recombinant Adeno Associated Viruses (AAV) are a commonly used gene delivery viral vector system to effectively introduce foreign genes into a broad range of cells and tissues. These gene delivery tools are widely explored in novel strategies for treating human diseases associated with blood, central nervous system and neuromuscular disorders or cancer. *In vivo* gene therapies approved in the US and Europe based on this approach have yet indicated minimal immunogenicity, minimal genome integration, and no pathogenicity. Previous exposure to naturally occurring AAVs results in pre-existing immunity that limits the efficacy of gene therapy and represents a potential safety concern. Therefore, it is important to screen for pre-existing AAV antibodies prior to gene transfer therapies. For most AAV gene therapy candidates currently in development neutralization assays are used to determine anti AAV antibody levels. These cell-based assays pose challenges such as lack of reproducibility, time consumption, and limitations in screening large sample numbers. An ELISA based method is an alternative that can be developed to measure total antibody levels against a specific serotype. In general, these types of *in vitro* assays are sensitive, reproducible, rapid, and can be validated to provide precise and accurate measurements even at high throughput demands. Therefore, we have developed an ELISA based anti-AAV antibody detection method using the automated GyroLab immunoassay platform that requires minimum sample volume while ensuring precision and accuracy of measurements. In this method, AAV capsid serotypes are immobilized on streptavidin-coated bead beds within the microcolumn of a GyroLab Bioaffy™ CD using AAV specific biotinylated ligands. The ratio of biotinylated ligands to form each specific complex is optimized. Primary antibodies against specific serotypes are captured by these immobilized AAV particles and detected by an Alexa Fluor 647-labeled secondary IgG antibody of the intended testing species. The method was qualified to detect anti-AAV8 antibodies between 7.8 ng/mL and 2000 ng/mL, anti-AAV9 antibodies between 16 ng/mL and 10000 ng/mL, and anti-AAV2 antibodies between 90 ng/mL and 25000 ng/mL, respectively. Anti-AAV antibody levels can be determined with ±20% accuracy of the nominal concentration and with ≤20% precision (%CV). These GyroLab immunoassays are capable of detecting and quantifying AAV antibodies in different matrices from multiple species such as human, mouse and non-human primates. Assay performance was determined using AAV positive and negative non-human primate sera obtained from a commercial vendor. Both calibrator standards and quality control (QC) samples are used for sample data acceptance within a single run. This established *in vitro* method can be utilized in preclinical studies to detect and quantify both pre-existing AAV antibodies as well as potential immunogenicity due to an AAV based gene therapy.



1012 Process Development for the Parallel Purification of Multiple Viral Vector Samples

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Adeno-associated virus (AAV) and lentivirus (LV) vectors have emerged as essential tools for gene and cell therapy applications. The purity of viral vector particles is critical for achieving effective transduction in cells while minimizing cytotoxicity. However, the purification of viral vectors is challenging when multiple vectors are processed simultaneously in parallel during the screening and selection of different vector genome configurations. A bottleneck to this approach is the current use of single-column chromatography systems that are not amenable to parallel processing. Therefore, there is a need to develop high-throughput chromatography methods capable of purifying multiple viral vector samples simultaneously in parallel to significantly reduce the overall labor, time, and resources. Our study uses the Protein Maker (PM) and AKTA PCC 75 that have been developed to provide an automated system capable of purifying multiple protein samples in parallel and to scout multiple purification strategies. The PM system has been designed and optimized to enable highly efficient, parallelized purification of proteins. For example, using the standard single-column 4-step single-channel method, the PM will purify up to 24 samples under one method in parallel, or 1-4 different samples on up to 6 different column types under 6 different method variations in parallel. AKTA PCC 75 is useful for the purification of up to 3 samples simultaneously. However, it has not been reported for AAV and LV vector purification using the PM or AKTA PCC 75. Here, we have used the PM and AKTA PCC 75 to purify multiple AAV and LV vector samples in parallel. We have produced AAV and LV vectors by transient transfection of HEK 293 cells using AAV-Max and LV-Max transfection reagents, respectively. Purification of these vectors was carried out by loading multiple vector samples in parallel onto their corresponding column using Protein Maker or AKTA PCC 75 separately. After elution, the vector samples were concentrated and formulated for gene therapy applications. The yields and purity of our viral vector samples were comparable to the conventional single-channel chromatography-based purification methods. Results from these successful studies demonstrate that a high-throughput viral vector purification procedure that can be useful for screening and candidate selection during process development and can be translated to Current Good Manufacturing Practices (cGMP) production for manufacturing clinical-grade vectors.

1013 Quantifying Indel Frequencies of Novel Nucleases with Unknown Cleavage Sites

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Nucleic acid-guided nucleases that edit polynucleotide sequences with high precision are characterized by their cleavage site relative to a target sequence and editing window. Existing next generation sequencing (NGS) analysis pipelines to quantify nuclease induced insertion and deletions (indels) assume that the cleavage site and editing window are established. However, for discovery and engineering of novel nucleases these properties may not be defined, which complicates editing analysis with standard tools. Here we establish Na-Lyashenko pipeline, a comprehensive and unified NGS-based workflow to characterize indels in a nuclease-agnostic manner. First, our pipeline sets rigorous QC metrics for NGS sequencing of the samples and visualizes read alignments to the amplicon. Second, we identify data-driven parameters for each sample depending on the combination of read length, amplicon prep, and nuclease to accurately quantify indels in the aligned reads. Thirdly, we customize parameters in a publicly available CRISPResso2 tool to quantify indels in a window centered on the target sequence and spanning +/-5 bp from each end of the sequence. We validated Na-Lyashenko pipeline with SpCas9 edited samples with a known target sequence and cleavage site (-3bp) against the default CRISPResso2 parameters. We then took highly edited samples (>80%) generated by a novel Cas12a nuclease with an unknown cleavage site and diluted defined ratios with non-edited samples. We found the observed indel percent of these mixed samples closely correlated with the expected indel percentage. Moreover, we leveraged Na-Lyashenko to quantify larger insertions (>10 nucleotides) with a 21-nucleotide library insertion and serially spiked-in a defined sequence. We found the observed spike-in sequence percent agreed with the expected spike-in percentage. The results demonstrate that the Na-Lyashenko pipeline can accurately quantify indels without the need for a defined cleavage site or specific editing window. Taken together the Na-Lyashenko pipeline may facilitate data analysis of indels to help characterize editing by novel nucleases.

1014 Insect Cell Baculovirus Expression Plaform for Large Scale Producton of rAAV

Sandra Bennun

uniQure, Lexginton, MA

Recombinant adeno-associated virus (rAAV) has become one of the main platforms for the development of novel human gene therapies. The ability to make high-quality vectors can be achieved using the baculovirus expression vector system using host insect cells. This approach offers a more scalable methodology to rAAV production potentiating their application for safe and cheap manufacturing. The successful scale-up production of rAAV vectors using the baculovirus expression system to supply the required genes is demonstrated at 500L scale. uniQure approach, based on Pilot Plant experience for large scale production of rAAV vectors in a Baculovirus expression vector/insect

cell system provides insights into the role of insect cell technology in the development and production of scalable manufacturing rAAV processes.

1015 Dose Control of rAAV Transgenes by Bioorthogonal Small Molecule Epigenetic Recruiters

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Adeno-associated virus (AAV) is one of the most frequently used vectors for the therapeutic transfer of genetic material. Current AAV gene therapy clinical trials seek to treat a diverse set of human diseases, from cancer to congenital genetic disorders. However, the application of AAV based therapies in some disease contexts is limited by insufficient transgene expression in targeted tissues. Our group has developed a chemically-based, bioorthogonal system that can epigenetically control the dose of genetic targets. The technology uses substrate-specific bifunctional small molecules, called chemical epigenetic modifiers (CEMs), to recruit endogenous epigenetic machinery to targeted genes. When paired with synthetic DNA-targeting modules such as dCas9 or zinc fingers (ZFs), CEM technology is capable of tuning genetic expression in a reversible and target-specific manner. To achieve dose control of AAV transgene expression using CEM technology, we developed a series of ZF-based, minimal autogenous regulatory cassettes that can be embedded into single-stranded vectors and retain 2.8-3.1 kb for therapeutic transgene payloads. We demonstrate that these autogenous CEM cassettes can efficiently control transgene expression in a CEM-dose dependent manner *in vitro*. In human cell lines, treatment with a bioorthogonal bromodomain containing protein 4 (BRD4) recruiter CEM induces AAV transgene expression by 10-fold. Upregulation of AAV expression by CEMs is shown to be reversible, to be CEM-dose dependent, and to scale with viral titer. This novel chemical approach to finely control and enhance expression of AAV transgenes may improve the safety and efficacy of future gene therapies.

1016 Single-Cell Transcriptional Analysis of Human *In Vitro* Platforms to Study Genome Editing Effects in Retinal Tissues

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Human stem-cell-derived retinal organoids offer powerful platforms for studying molecular programs associated with normal and disrupted eye function as well as for rapid assessment of potential targets of genome editing. The efficiency of genome editing in traditional 3D organoid systems is limited because of the low rate of transduction with non-AAV based delivery platforms. We observe that this transduction efficiency could be enhanced by removing the extracellular barriers for viral vector delivery via organoid dissociation and replating the cells into a 2D culture for transduction. However, it is unclear whether 2D platforms faithfully capture the cell type composition and gene expression programs of 3D organoids. To compare the transcriptome states of cells in 2D dissociated cultures and 3D organoids, we performed single cell RNA sequencing (scRNA-seq) of the two different platforms by taking samples from six different time points corresponding to different stages of retinal cell fate specification. To enable integrative analysis of this complex multi-sample dataset, we developed and applied a novel multi-task matrix factorization method named Tree-guided non-negative Matrix Factorization (TMF), which performs sample-specific dimensionality reduction while incorporating the relatedness of the samples. TMF identified cell clusters that were enriched in different retinal cell types. Both 2D and 3D platforms show the presence of a diverse set of cell types including Müller glia, photoreceptors, and horizontal cells. We identified a total of 22.4% of cells from 2D cultures as photoreceptors compared to 51.2% of cells from the 3D organoid cultures, while 2D cultures had a higher proportion of RPE/progenitor cell types compared to 3D cultures. Photoreceptors identified in either platform show expression of known cone and rod marker genes, which validate the identity of these cell types. The photoreceptor cells maintain functionality, as they maintain visual cycle function after delivery of the genome editor. Additionally, we compared transcriptional profiles of transduced cells with that of untransduced cells. In total, 42% of 2D cells were transduced, of which 8% comprised photoreceptors. Overall, this work shows that 2D platforms retain a sizable photoreceptor population which can be efficiently transduced, making them an effective platform for examining molecular signatures of genome-editing in a higher throughput manner.

1017 *In Vivo* Delivery of Genetic Payloads to Human Hematopoietic Stem/Progenitor Cells

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Achieving in vivo genetic engineering of hematopoietic stem/progenitor cells (HSPC) would dramatically expand patients' access to gene therapy. However, preclinical development is complicated by the fact that mouse hematopoiesis differs from human and it is not permissive to all human-compatible vector pseudotypes. We have developed a strategy to assess in vitro and in vivo access to human HSPC and achieved efficient in vivo genetic engineering of naïve human HSPC using lentiviral (LV) using BaEVTR and retargeted fusogens. We initially conducted a high-resolution characterization of access to unstimulated HSPCs and resting individual HSPC subtypes in vitro and observed superior BaEVTR targeting of HSPC (~100% at MOI 66, with Vectofusin-1) compared with VSV-G (40% at MOI 50,750), as well as ~20% transduction with BaEVTR LV of resting FACS-sorted hematopoietic stem cells (HSC) at MOI 2, a dose approaching in vivo scenarios. We then set to establish basal access to human HSPC in the bone marrow (BM) and peripheral blood (PB) without relying

on high vector doses or selective enrichment. First, we injected intravenously (IV) BaEVTR and VSV-G LV in long-term humanized mice and showed that 1 low dose BaEVTR LV could transduce 2-4% BM multipotent progenitors vs no detectable transduction in that population using same doses of a VSV-G LV. Because stably humanized mice host skewed human HSPC composition, we characterized early human engraftment and injected LV at D7 of humanization, a timepoint at which we established that human cells are localized only in the BM and the HSPC composition still mirrors the infusion composition, thus providing a unique system to isolate and study in vivo BM access to human HSPC. Mobilization (G-CSF/AMD3100) at this early stage of humanization did not result in substantial egress of human HSPC into the PB but, intriguingly, unlocked transduction to naïve HSC in the BM allowing ~2% targeting of phenotypic HSC with BaEVTR LV compared to undetectable transduction without mobilization. To conversely isolate and study basal PB access to HSPC, we infused mobilized CD34+ cells into NBSGW mice immediately followed by IV injection of LV. In this setting, 1 low dose BaEVTR LV reached 23% transduction of Lin-CD34+ cells and 12% of phenotypic HSC measured at D12 in the BM. This resulted in a 21-23% GFP+ human PB myeloid output over the first 6wks after infusion, stabilizing at 3-7% by wk9. The mice were later mobilized at 11wks and injected with another set of LV carrying the RFP reporter gene to measure stability and plasticity of engineered engrafted HSC as well as susceptibility to multiple transductions. Together the BM and PB access models provide a system to quantitatively assess in vivo delivery to HSPC. In considering bioavailability in the presence of other cellular "sinks," we tested BaEVTR LV transduction of primary human hepatocytes and observed that BaEVTR shows substantially lower gene transfer rates when compared to VSV-G, suggesting a potential evasion of the liver upon in vivo delivery in humans. To reduce off-target delivery, at SANA we are developing retargeted fusogens that are engineered to recognize receptors on chosen target cell types resulting in highly cell-specific in vivo gene transfer. We evaluated retargeted fusogen LV via IV injection and showed a striking 100x specificity increase over other broadly tropic pseudotyped LV in targeting a receptor-positive population exclusively localized in the BM, which make up as little as 0.15% of total human cells. Overall, we generated a robust strategy for establishing basal access and achieving efficient specific in vivo delivery of genetic payloads to human HSPC.

1018 Evaluating the Potential of CRISPR Activation Therapy to Increase *TCF4* Expression in Pitt-Hopkins Syndrome

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Pitt-Hopkins Syndrome (PTHS) is characterized by severe intellectual disability and tendency for epilepsy and/or hypotonia. There is currently no treatment for PTHS. PTHS is caused by *de novo* mutations in *Transcription Factor 4* (*TCF4)* which typically result in haploinsufficient expression. *TCF4*, which codes for a basic helix-loop-helix transcription factor, exhibits high expression in the central nervous system. *TCF4* appears to be critical for neuronal development and neuronal function. Cre-induced knockout of *TCF4* in adult mice altered firing patterns and dendritic structure in excitatory neurons, and induced memory deficits. However, the effects of PTHS may be ameliorated by restoring *TCF4* expression. Cre-induced knockout of a STOP cassette in the Tcf4 gene in mice, to reinstate Tcf4 expression in a PTHS model, improved activity levels, memory, and innate behaviors such as nest building. *TCF4* expression reinstatement also normalized EEG abnormalities and expression of key *TCF4*-regulated genes related to neuronal maintenance. It is thus plausible that rescue of standard *TCF4* expression could be beneficial. CRISPR activation (CRISPRa) is a valuable avenue of expression rescue by targeting the wild-type promoter of *TCF4* for activation. CRISPRa is comprised of a guide RNA (gRNA) to target the promoter of the gene, a deactivated Cas9 (dCas9), and tethered transcriptional activators. We evaluated gRNAs for human *TCF4* to design a CRISPRa system.**Methods**: 23 gRNAs designed for the human genome were screened in HEK293T-LentiX cells. Of the guides, nine were designed from a dataset based on a predictive algorithm and 14 were designed from the ChopChop software. The gRNAs were cloned into a codon optimized gRNA expression plasmid. The gRNA plasmid was co-transfected with two other plasmids containing one half each of the dCas9-VPR-MPH complex, separated by an intein sequence. After analysis, a set of candidate lead gRNAs was selected and the same transfection method was performed on two independent iPSC-derived neuronal stem cell (NSCs) lines. In addition, patient (*TCF4* KO) iPSCs and same-sex parental control iPSCs were differentiated into NSCs, for lead gRNA evaluation.**Results:** From the HEK293T transfection (for human guides) five gRNAs exhibited significant upregulation of *TCF4* transcript expression. The lead guide was chosen as the guide with the greatest expression fold change from the transfection of the five guides in the healthy and patient NSCs. **Conclusion:** CRISPRa is able to significantly upregulate *TCF4* across multiple cell lines. CRISPRa will be tested in multiple PTHS cell lines to evaluate functional rescue. As transduction can be a more efficient method of therapy delivery compared to transfection, transduction experiments with the lead guide will be performed.

1019 High-Throughput Functional-Based Screening of Chimeric Antigen Receptor (CAR)-T Cells Using Nanovial Technology

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We present a workflow that leverages flow cytometry and cavity-containing hydrogel microparticles to enrich antigen specific chimeric antigen receptor (CAR)-T cells and sort functionally active populations in high-throughput based on cytokine secretion. CARs are synthetic immune receptors that repurpose natural signaling components to redirect T cells to selectively eradicate cancers but have shown limitations including toxicities, in vivo persistence, and tumor relapse. In addition, the limited success of the CAR-T approach in solid tumors has been generally attributed to a hostile tumor microenvironment (TME). Sub-optimal design of current CAR constructs also plays a key role. The lack of an efficient and accessible functional-based method for interrogating CAR-T cells impedes the development of T-cell-based immunotherapies. To address this gap, we developed an

approach to screen millions of functionally active CAR-T cells using cavity-containing hydrogel microparticles (Nanovials). Nanovials act as suspendable sub-nanoliter containers for individual cells that can be modified to perform biological assays. Here we modified nanovials with a model antigen (CD19) to enrich and bind individual antigen-specific CAR-T cells into the cavity of the nanovials. Nanovials are modified with antibodies against target cytokines to capture secreted cytokines (IFN-gamma, IL2, TNF-alpha) from activated CAR-T cells. Captured cytokines stained with fluorescent secondary antibodies to identify secreting cell populations and nanovials containing functionally active CAR-T cells are then analyzed and sorted using standard commercially available flow cytometers. Cells isolated after flow can then be expanded for further studies or sequenced to recover transcript information. Using this workflow, we quantified the amount of cytokine secreting CAR-T cells across different patient samples and correlated cytokine secretion levels to differences in CAR engineering. Furthermore, we identified and isolated significantly different subpopulations of CAR-T cells within a patient sample on the basis of cytokine secretion. The capability to resolve and isolate subpopulations within a single patient sample is made possible through the single cell resolution enabled by our platform and is not possible through current bulk analyses techniques such as ELISA, or other advanced technological readouts like impedance based cell-killing assays.In summary, we present a flow cytometry approach to screen CAR-T cells based on antigen specificity and cytokine secretion using our nanovial platform. This readily adoptable platform can provide researchers with improved tools for screening CAR designs at scale and can be used to improve current quality control workflows used in cell therapeutic manufacturing.

1020 rAAV Gene Therapy for Fabry Disease: Enhanced Secretion of α-GLA by Signal Peptide Engineering

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Fabry disease is an X-linked lysosomal storage disease due to a deficiency of α-galactosidase A (α-Gla A) with the prevalence of approximately 1 in 40,000.There are two clinical subtypes of Fabry disease, classic (type I) and late-onset (type II), depending on the level of enzyme function in patients. A typical characteristics of this disease is the progressive and systemic accumulation of sphingolipids such as globotriaosylceramide (Gb3) and its derivative, globotriaosylsphingosine (lyso-Gb3), in the lysosomes of vascular endothelial cells. The standard treatment for patients is enzyme replacement therapy which is a lifelong burden and a considerable proportion of type I males eventually produce neutralizing antibodies. The overexpression of α-Gla A in the liver by rAAV gene therapy is believed to be a longer lasting and effective treatment. Thus, we hypothesized that the use of an α-Gla A gene with enhanced secretion capacity compared to the wild type may have better treatment efficiency. To this end, the signal peptide of α-Gla was replaced by 22 heterogenous ones and the constructed plasmids were used to transfect HepG2 cells individually. The supernatants were collected and used for ELISA and enzymatic assays. During the screening, four signal peptides were found to have 1.5-3 folds higher expression levels and enzyme activities than the wild type. sp21 was further evaluated in vivo using Fabry mice. A codon optimized α-Gla A with the sp21 peptide under the control of a novel liver specific promoter were packaged in a self-complementary rAAV vector with serotype AAV8. Two doses of the vector, $2x10^{12}$ and $5x10^{12}$ vg/kg, were administrated by tail vein injections. α-Gla A activity levels were measured in plasma sampled at 0, 2, 4, 6, and 8 weeks. Expression levels were stable since week 2 with activities as high as 1678 nmol/h/mL. Mice were sacrificed at 6 and 14 weeks to quantify lyso-Gb3 level in plasma by LC-MS/MS. We demonstrated that the lyso-Gb3 was largely cleared in the plasma and the amounts were less than 1%. It was also demonstrated by IHC that the expression levels of α-Gla A in the liver, heart, spleen, and kidney at week 14 were significantly higher than the negative control.

1021 Next-Generation of AAV Gene Therapy for Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder caused by a mutation in the survival motor neuron 1 (*SMN1*) gene, which leads to progressive muscular weakness and hypotonia as a consequence of the loss of α motor neurons of the spinal cord anterior horn. Gene therapy has been successfully developed for SMA and the commercialized medicine Onasemnogene abeparvovec (Zolgensma), was approved by FDA in 2019 as a one-time intravenous (IV) administration to SMA patients below the age of 2 years, which demonstrated significant clinical benefits including prolonged survival and motor milestone achievement. However, current gene therapy faces various challenges including insufficient expression of the *SMN1* gene in the target organs/tissues and off-target toxicity such as liver failure. Thus, there is a need for improved gene therapies for SMA with tissue specific SMN expression and reduced off-target toxicity. Our first-generation product of AAV-based gene therapy (EXG001-307K) had demonstrated a better extended survival, greater motor improvement and significantly reduced toxicity in the mouse model of SMA and a very promising profile in our clinical trials. To further enhance the efficacy and safety, we are designing the second-generation gene therapy product including AAV novel constructs with optimized transgenes and new route of administration (ROA). Codon optimized transgenes, secreted forms and stabilizing mutants of human *SMN1* (*hSMN1*) have been extensively studied both *in vitro* and *in vivo*, including the comparison of protein expression level, potency, secreting pathway, and efficacy. Significantly higher protein expression level has been achieved from the novel transgenes compared to the natural sequence of *hSMN1*, and a subset of the candidates showed a better survival rate and behavior improvement than the first-generation product EXG001-307K. In addition, we have also examined direct intra-CSF administration (new ROA) of EXG001-307K in SMNΔ7 mouse model, which revealed higher AAV genome biodistribution and protein expression in the central nervous system (CNS) and a lower distribution and protein expression in the peripheral organs (liver and heart) thus a better profile of efficacy and safety (lower off-targeting) than the same dose when administered by IV. Transduction efficiency and dose escalation have also been assessed and compared in mouse and nonhuman primates (NHPs) to assure a feasible and efficacious dose range for the translational work towards clinical application.

1022 PDL1 and CXCR5 Modified FVIII Specific TRuCε Tregs Exhibit Enhanced Trafficking and Suppression of Anti-Drug Antibody Formation

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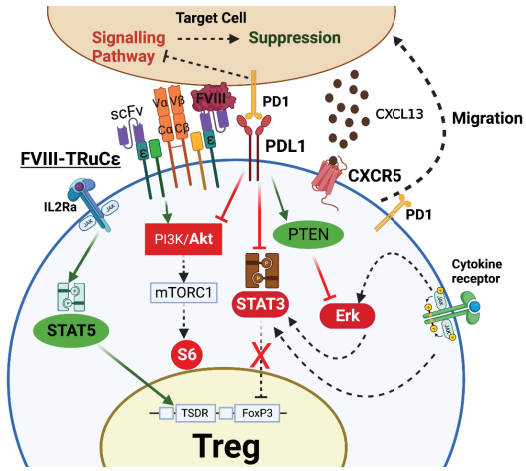
Anti-drug antibodies (ADA) can develop against therapeutic biologics, reducing treatment efficacy and leading to unwanted side effects. In hemophilia A (HA), which is an X-linked blood clotting disorder caused by mutations in the factor VIII (FVIII) gene, replacement protein therapy with recombinant FVIII is significantly complicated by ADA formation. Previously, we established that cellular therapy with engineered FVIII specific T cell receptor fusion construct (TRuC) regulatory T cells (Tregs) delivers specific and durable suppression of ADA responses in a preclinical HA model [Mol Ther 2021, 29:2660-2676]. Here, we engineered FVIII TRuCε Tregs to co-express the programmed death 1 ligand 1 (PDL1) checkpoint inhibitor or the chemokine receptor CXCR5, with the aim to improve suppressive capacity, phenotypic stability, and in vivo localization to germinal centers, which are the site of ADA development. First, we validated a role for PDL1 in ADA tolerance by showing that 2 intraperitoneal injections of non-lytic mouse PD-L1Fc (100 µg/mouse) administered 1 day prior to FVIII weekly injections (1.5 IU/week for 4 weeks) caused HA mice to develop 3-fold lower ADA titers (5.16±4.7 BU/mL) compared to mock treated animals (18.15±6 BU/mL). In vitro, we confirmed that FVIII TRuCε-PDL1 and FVIII TRuCε-CXCR5 expressing murine Tregs responded specifically to FVIII stimulation by upregulating CD69, PD1, ICOS, CTLA4 and LAP. PDL1 signaling significantly downregulated phosphorylation of STAT3, Akt (S467), S6 and Erk, whereas STAT5 phosphorylation in response to FVIII stimulation was unaffected. Therefore, PDL1 acts at the level of PI3K/Akt, RAS/MAPK and STAT3 signaling to conserve the immunosuppressive phenotype of FVIII TRuCε-PDL1 cells via a process of suppressive "back-signaling" (**Figure**). Next, we confirmed enhanced chemotaxis of FVIII TRuCε-CXCR5 Tregs towards a CXCL13 gradient, which was generated across a transwell in vitro or by continuous FVIII injections in vivo. Finally, adoptive transfer of 5 x 10^5 FVIII TRuCε-PDL1 or FVIII TRuCε-CXCR5 Tregs was able to control ADA formation in response to weekly FVIII injections in HA mice. 8 weeks following adoptive transfer, control mice that received only FVIII injections developed high titer ADAs of 14.36 ± 5.536 BU/ml. FVIII TRuCε-PDL1 and TRuCε-CXCR5 Treg recipient animals had significantly lower ADA titers of 2.501 ± 1.081 BU/ml (p=0.003) and 1.963 ± 0.7442 BU/ml (p=0.0169), respectively. Suppression was maintained for up to 12 weeks in the FVIII TRuCε-PDL1 recipient group as compared to the FVIII control group (7.702 ± 2.185 vs 34.32 ± 9.052 BU/ml, p=0.023), demonstrating that co-expression of PDL1 prolongs the effectiveness of engineered Treg therapy. This study shows that the PDL1/PD1 axis is important for promoting the suppressive capacity of FVIII TRuCε Tregs, whereas expression of CXCR5 improves recruitment of engineered T cells to sites of immune response initiation. We are currently evaluating the efficacy of a combined FVIII TRuCε-PDL1-CXCR5 receptor construct and defining the immune mechanisms underlying its enhanced suppressive capacity.



1023 ADI-270: An Armored Allogeneic "Off-the-Shelf" CAR γδ T Cell Therapy Targeting CD70+ Cancers

Gauri Lamture, Alexander G. Teague, Morgan Smith-Boeck, Michael Salum, Ramandeep Kaur, Smitha Rao Yelaguli Gundurao, Pannaga Parthasarathy, Amy Doan, Jyothi Sethuraman, Ngoc Hoang, Hayden Tessman, Philip Storm, Soojin Han, Swapna Panuganti, Chris Rold, Yvan Chanthery, Marissa Herrman, Blake T. Aftab, Arun Bhat, Kevin P. Nishimoto

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Background: CD70 represents a compelling target for the development of CAR T cell therapies due to its high expression in multiple solid and hematological malignancies. CAR T efficacy in solid tumors has been a key challenge in the field and emerging strategies to improve clinical responses is to employ alternative cytotoxic effector cells with multifunctional tumoricidal activity. γδ T cells combine innate and adaptive immunity to kill malignant cells, and their infiltration into various cancers, including those expressing CD70, significantly correlates with survival. Strategies targeting CD70 by engineering its natural receptor (CD27) as the antigen-recognition moiety of a CAR have demonstrated superior preclinical antitumor activity compared to scFv-based approaches. Additional armoring of CAR-T cells to mitigate the immunosuppressive tumor microenvironment can further enhance activity. Here we report the manufacturability and functionality of ADI-270, a γδ T cell product expressing CD27 natural receptor CAR armored with a dominant negative TGFβRII, for targeting CD70+ cancers. **Methods:** Qualified donor PBMCs were used to activate, expand, and engineer Vδ1 T cells to express CD70 CAR in an established manufacturing process. *In vitro* phenotype, cytokine profile, and antitumor functionality of ADI-270 were determined using flow cytometry, multiplex cytokine assays, and cell-based cytotoxicity assays against cell lines having a wide range of CD70 expression. Human tumor xenograft models in immunodeficient mice were used to evaluate *in vivo* efficacy after a single dose of ADI-270. In addition, *ex vivo* analysis was performed to evaluate the proliferation and phenotype of ADI-270 cells in the tumor and non-tumor tissues. **Results:** Vδ1 T cells modified to express CD70 CAR were successfully generated and expanded, indicating product expansion was not hindered by putative

risks for CD70-mediated fratricide in the manufacturing process. The resulting ADI-270 Vδ1 CAR T cells expressed a predominant naïve-like memory phenotype with potent *in vitro* cytotoxicity and production of proinflammatory cytokines against CD70+ tumor cell lines. Lastly, ADI-270 significantly inhibited tumor growth *in vivo* and proliferated specifically in tumors. **Conclusion:** In summary, ADI-270 demonstrates preclinical proof-of-concept of an armored allogeneic CD70 γδ CAR T cell therapy utilizing the CD27 natural receptor CAR format for targeting CD70^{+} cancers. These data support continued development and further investigation of ADI-270 in the clinic.

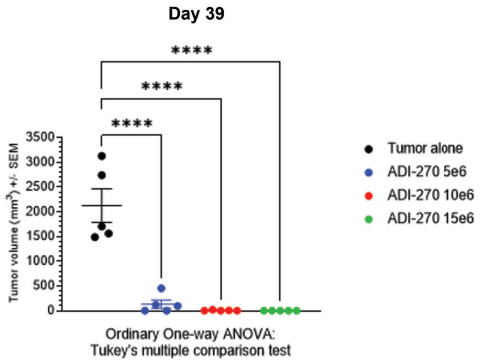

Figure 1: In vivo efficacy of ADI-270 in a subcutaneous A498 tumor model in NSG mice
The average tumor volumes for the duration of the study and statistical comparison between treatment groups and the tumor-alone control group at the end of the study (Day 39).

1024 Liver Fibrosis Alters AAV Biodistribution and Impairs Hepatocyte Transduction

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Adeno-associated viral vectors (AAVs) are the most promising tools for liver-directed gene therapy. However, integrity of hepatic architecture has been considered pre-requisite for efficient gene delivery and patients with hepatic damage and fibrosis are currently excluded from clinical trials. However, AAV-mediated gene transfer to fibrotic livers has been poorly investigated so far. In the present study, we investigated biodistribution and hepatocyte transduction of AAV8-based vectors, commonly used in liver-directed gene therapy clinical trials, in the context of liver fibrosis. Analysis of three mouse models of induced and genetic liver fibrosis revealed that fibrotic livers were transduced less efficiently by AAV8 and this resulted primarily from reduced vector uptake by the liver, rather than from vector genome loss due to hepatocyte proliferation. Moreover, liver fibrosis altered vector blood clearance and biodistribution in extrahepatic organs, significantly increasing viral particle uptake by spleen and kidney. AAV immune profile and distribution in liver and spleen at the cell-type level in fibrotic animals are currently under investigation. Overall, these findings demonstrate that liver fibrosis impairs AAV-mediated gene transfer to hepatocytes and highlight the relevance of the limitations posed by liver fibrosis to efficient and safe gene transfer.

1025 AAV-CRISPR/Cas9 Mediated Gene Editing in Patient *RPGR* hiPSC-Derived Retinal Organoids

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Background: Mutations in the *RPGR* gene are the predominant cause of X-linked retinitis pigmentosa (XLRP). An inherited retinal dystrophy with a progressive loss of photoreceptors leading to eventual blindness. Notably, RPGR has two primary protein isoforms, the constitutive RPGR$^{1\text{-}19}$ protein translated from 19 exons expressed in most tissues, and the retinal specific RPGR^{ORF15} protein translated from the first 14 exons plus a final exon 15 and part of intron 15 (ORF15). The *RPGR* ORF15 isoform is differentially spliced to terminate in intron 15, a highly repetitive region and known mutational hotspot. The exact function of RPGR^{ORF15} in the retina has not been fully elucidated, but it is known to localise in the transition zone of the photoreceptor cilium and presumably plays a role in protein trafficking and outer-segment maintenance. **Results:** iPS cells were generated from *RPGR* patient skin fibroblasts (c.706>T p.Gln236* and c.425T>G p.Ile142Ser) with nonsense or missense mutations, respectively. Corresponding isogenic controls were generated by CRISPR/Cas9. The *RPGR* and isogenic control hiPSC were validated by sequencing whereas absence of genomic instability was checked by copy number variation tests and karyotyping. In order to effectively model *RPGR* XLRP, the *RPGR* patient hiPSC lines and their isogenic controls were differentiated into retinal organoids. Retinal organoids were cultured up to differentiation day 240 and characterised by immunohistochemistry, examining phenotypic variations in photoreceptor and cilial morphology. Additionally, functional RPGR^{ORF15} is post-translationally modified in the highly-repetitive C-terminal region by glutamylation. Upon differentiation to retinal organoids, both patient lines exhibited a loss of glutamylated RPGR^{ORF15} as determined by Western blot. Currently there is no known cure for *RPGR* XLRP, although progress has been made with AAV delivered gene augmentation strategies. As an alternative approach, we have pursued the development of a broadly applicable AAV-CRISPR/Cas9 mediated gene editing strategy. The post-mitotic nature of the retina necessitates a gene editing strategy that does not utilise homology directed repair, as this pathway is not active in non-dividing cells such as photoreceptors. Here, we use a homology-independent targeted integration (HITI) based strategy to insert a codon optimised super-exon, including exons 2-ORF15 of *RPGR* with a C-terminal self-cleaving EGFP reporter (4.4 kb). By targeting intron 1 of *RPGR* we are able to replace 99.9% of the coding sequence of the *RPGR* gene whilst retaining the endogenous promoter. The feasibility of this gene editing strategy has been demonstrated in HEK 293T cells, with 5' and 3' junction PCR confirming desired integration of the donor sequence in the *RPGR* intron 1 locus. As of yet, we are still to quantify the efficiency and precision of this strategy in hiPSC-derived retinal organoids. **Summary/Discussion:** We successfully generated patient *RPGR* hiPS cells and their isogenic controls. We differentiated these hiPSC into retinal organoids, and at first glance there are no major histological differences between *RPGR* mutant and wild-type retinal organoids. We aim to deliver a CRISPR/Cas9 system to retinal organoids via dual

AAVs, with one AAV containing the gene editing machinery (SaCas9 and sgRNA), and the other delivering the 4.4 kb donor sequence. Upon successful gene editing in retinal organoids we will determine the correct expression, localisation and post-translational modification of RPGR.

1026 Development of an alpha-Synuclein Targeting Vectorized Antibody Strategy: First *In Vivo* Proof of Mechanism in Wild Type Mice

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Synucleinopathies are neurodegenerative disorders characterized by aggregation of alpha-synuclein protein (α-Syn), encoded by the SNCA (Synuclein Alpha) gene. Alpha-synuclein is expressed in the brain and also peripherally in red blood cells. In the brain, in the context of neurodegenerative disease, α-Syn can form aggregates, which in turn may spread in a prion-like manner, with toxic aggregated forms being transmitted from cell-to-cell as the disease progresses. In this regard, facilitating the clearance of α-Syn aggregates outside of the cell may have therapeutic potential by reducing the spread and potentially slowing or even halting disease progression. Hence, passive immunization with α-Syn-targeting antibodies is in focus of many ongoing preclinical and clinical studies. The main challenge of administering α-Syn antibodies peripherally is for them to pass through the blood-brain barrier to perform their function. Additionally, α-Syn antibodies will bind to α-Syn that is abundant in the blood resulting in a sudden decrease in their bioavailability. Lastly, repeated antibody injections will be needed for a sustainable antibody therapy, which can lead to anti-drug antibody formation. We are developing specific α-Syn vectorized antibodies, using our AbQURE™ technology platform, to provide a stable supply of α-Syn antibodies in the brain. Full length α-Syn antibodies AbQURE-1 and AbQURE-2, both targeting the C-terminal part of α-Syn, were successfully expressed in our AbQURE™ platform, both from a plasmid and from an AAV (AAV-AbQURE-1 and AAV-AbQURE-2). We established proof of mechanism (PoM) in wild type mice after direct brain administration of the vectorized antibodies. In this study, we showed a dose-dependent expression of the antibodies in the brain, using both biochemical and histological methods. Detection of antibodies with in-house developed MSD assays showed functional vectorized antibody expression in the striatum. This was confirmed by immunohistochemical methods in relevant tissue sections. No safety concerns were reported in the pathological examination of the Hematoxylin and Eosin (H&E) staining of the treatment groups compared to the negative control group. Present data demonstrates a safe and efficacious expression of specific α-Syn antibodies using our proprietary AbQURE™ platform. As a follow up, efficacy of anti-α-Syn antibodies to reduce the α-Syn spreading will be evaluated in relevant disease models in the context of therapeutic application to synucleinopathies. *AbQURE is a trademark registered in the European Union and United Kingdom and pending in other jurisdictions.*

1027 Establishment of a Clonal, Serum-Free, Suspension-Adapted HEK293 Cell Line for the Manufacturing of Adeno-Associated Virus Vectors

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Adeno-associated virus (AAV) is an effective viral vector for gene therapy due to several factors: infection of post-mitotic cells, non-pathogenic, broad tissue specificity coupled with efficient cellular transduction, and durable transgene expression. As AAV-based gene therapies are becoming increasingly more prevalent, a renewed emphasis on scalable manufacturing platforms efficient in generating high yields of, highly pure, and potent quantities of viral vectors is paramount to success. Moreover, these scalable processes should focus on the overall reduction in costs-of-goods to make these complex therapies self-sustaining and accessible to all. Utilizing the well-established, adherent, Frank Graham HEK293 cell line as a starting point, we have successfully adapted cells from a qualified master cell bank to grow in animal component-free, suspension conditions, in shake flasks and bioreactors. In head-to-head studies comparing our CBM-HEK suspension-adapted versus 3 commercially available suspension HEK293 cell lines, we demonstrate similar, if not better, yields of AAV vectors, following plasmid triple transfection method for virus production. To achieve this, we focused specifically on a number of parameters to optimize such as media selection, transfection reagent optimization, transfection parameters and cell density. In order to establish a cell line amenable for use in GMP-grade manufacturing, we clonally printed CBM-HEK293 pool cells using the Cytena f.sight cell printing and CloneSelect imaging platform. Herein, we show full-traceability of clonal outgrowth from single-cell through scale-up and have identified select clones that perform equally well in head-to-head comparison studies versus other HEK293 cell lines for virus production. Moreover, these CBM-HEK293 clones can support the production across a diverse panel of all natural AAV serotypes. We are currently establishing a GMP master cell banks for use in large scale AAV manufacturing.

1028 Highly Specific Interaction of Exosomes with Long-Term Hematopoietic Stem Cells

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Long-term hematopoietic stem cells (LT-HSCs) have been of high interest due to their exceptional self-renewal potential, persistence, and capability to repopulate all blood cell populations. This is very important in terms of bone marrow transplantation, and gene therapy/gene editing approach where transplanting a sufficient number of LT-HSCs or therapeutic gene modification in these cells can guarantee the

success of treatment. LT-HSCs are a rare population of hematopoietic stem and progenitor cells (HSPCs). Generally, HSPCs are identified with CD34 surface marker and contain multiple other populations such as long-term hematopoietic stem cells (LT-HSCs), multipotent progenitors (MPPs), and common myeloid progenitors/granulocyte-monocyte progenitors/megakaryocyte-erythroid progenitors (CMP/GMP/MEP) that all require a long list of surface markers for hierarchical organization. Previously it was shown that a panel of 5 antibodies targeting surface markers on HSPCs could differentiate LT-HSCs ($CD34^+$, $CD38^{-/low}$, $CD90^+$, $CD45RA^-$, $CD49f^+$) from other lineages [1]. However, to date, there has not been any report on a single molecule capable of identifying LT-HSCs. Our recent data show that using small nanovesicles (30-100 nm) called exosomes we can achieve almost 80% targeting of LT-HSCs. We performed large exosome screening from different cancer types and healthy tissues and we identified exosomes that highly interact with LT-HSCs. Especially cancers with bone metastatic profiles had higher LT-HSC interaction. A perfect example of this was prostate cancer exosomes from two different PC3 (low bone metastasis profile) and C4-2B (high bone metastasis profile) cell lines in comparison to normal human primary prostate epithelial cells (HPrEC). We saw five times more interaction of C4-2B exosomes with LT-HSCs (78.7%) in comparison to PC3 exosomes (16.2%) and HPrEC exosomes (3.6%). We achieved the same results for the H929 cell line which is a representative cell line for multiple myeloma. However, for ANBL6, which is another model cell line of multiple myeloma we did not see any significant interaction. This highly specific exosomal interaction in multiple myeloma could be related to lytic bone damage in this cancer type, where exosomal interaction with LT-HSCs might drive the osteoclast/osteoblast imbalance leading to lytic bone damage. Another important result of our study was discovering the highly specific interaction of normal human primary small airway epithelial Cells (HSAEC) with LT-HSCs. This specific interaction is happening with exosomes derived from healthy lung cells and shows the ongoing exosomal communication between lung tissue and LT-HSCs. It was previously reported that the lung contains hematopoietic progenitor populations however, this is the first time we report highly specific exosomal interaction between the lung and LT-HSC population. Following 48 h interaction of lung exosomes with CD34 cells we observed high colony-forming potential and higher CFU-GEMM colony formation. Currently, we are continuing our studies by understanding the relationship between bone metastasis and exosomal interaction with LT-HSCs. Also, we are doing in vivo studies to understand exosomal communication between lung exosomes and LT-HSCs. Our findings show a network of communications that both cancer cells and healthy cells use to modulate hematopoiesis and potentially promote metastasis in cancer.

References 1. Notta, F., et al., Science, 2011. 333(6039): p. 218-21.

1030 A Gene-Independent Treatment Strategy for Inherited Retinal Diseases Using Cas9-VPR-Mediated Cellular Reprogramming and Neuroprotection

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Inherited retinal dystrophies (IRDs) comprise a heterogeneous group of eye diseases which often affect rod photoreceptors and lead to retinal degeneration. Currently, there is no therapy to halt or slow down retinal degeneration for the vast majority of IRD patients. Most studies investigating therapeutic approaches for IRDs take advantage of the classical gene supplementation strategy using adeno-associated viral (AAV) vectors to express an intact copy of the defective gene in target cells. However, this therapy requires a comprehensive preclinical development and clinical trials for every individual gene and is hardly feasible given the large number of IRD-linked genes. Alternatively, inactivation of cell-damaging genes or activation of cell-protective genes could lead to a mutation- and gene-independent therapy approach that prolongs cell survival and could even stop disease progression. Recent approaches have shown that knockout of the *Nrl* gene can ameliorate the retinal phenotype in mouse models carrying mutations in rod-specific genes, by reprogramming rods into cone-like cells. Similarly, other studies have demonstrated that the delivery of one or both of the rod-derived cone viability-factors (RdCVF1 or RdCVF2) encoded by the nucleoredoxin like 1 or 2 gene (*Nxnl1 or Nxnl2*) to photoreceptor cells can improve the retinal phenotype in IRD mouse models. Nevertheless, both gene-independent strategies, while promising on their own, would benefit from an approach that combines their advantages. Here, using a multiplexing gene editing approach, we set out to establish a proof-of-principle for a novel gene-independent retinal gene therapy approach, based on concurrent knockdown of *Nrl* and activation of *Nxnl1* and *Nxnl2* in the retina. For this purpose, we applied dual mRNA trans-splicing AAV8 Y733F vectors (6×10^{11} vg/µl) to subretinally deliver the Cas9-VPR module in wild type animals and in the common mouse model for autosomal dominant retinitis pigmentosa ($Rho^{P23H+/-}$). Cas9-VPR expression was driven by a photoreceptor-specific rhodopsin kinase (GRK1) promoter and combined with sgRNAs of different spacer lengths targeting *Nxnl1*, *Nxnl2* (14 nt each) and *Nrl* (20 nt) to achieve gene activation or knockout, respectively. Four weeks upon subretinal injection, we confirmed *Nrl* knockout, successful reprogramming and activation of *Nxnl1* and *Nxnl2* in the murine retina using western blotting and qRT-PCR experiments. Optical coherence tomography and electroretinography in $Rho^{P23H+/-}$ mice demonstrated improved retinal structure and function up to six months after treatment. Taken together, we provide the first evidence for therapeutic benefit of a novel, gene-independent strategy for IRDs that utilizes concurrent Cas9-VPR-mediated knockout and activation of different genes designed for cellular reprogramming and neuroprotection. Many IRD-patients could profit from such an approach, but similar strategies could also be applied to non-ocular diseases.

1031 Drug Product Development of Adenovirus: Platform Approaches to Select Early Phase Formulation and Fill/Finish Strategy

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The gene therapy is an accelerating development field utilizing many types of viral vectors to deliver the gene of interest. One of these vectors is adenovirus which is typically produced using suspension or adherent upstream process. Both platforms can produce a high titer (>1E12 vp/mL) adenovirus drug substance. Challenges arise when proceeding through the downstream purification steps and into the final drug product process steps. In this study, a high titer adenovirus drug substance was used as the basis for a drug development campaign that encompassed both formulation confirmation and fill/finish development. The target formulation of interest was adapted from previous literature and compared to a base buffer to demonstrate feasibility of meeting target product profile. Here we demonstrate that a specific set of analytical methods targeting physical, colloidal and physical titer attributes is capable to decipher a more optimal formulation with a small material requirement in an expedited manner. Additionally, a fill/finish process was designed using a small-scale V-max study to select appropriately sized and compatible sterile filters. The resulting data from particulates, turbidity, and viral genome titer measurements allowed to minimize product loss due to material adsorption and hold-up volume and prevent a catastrophic event of filter fouling.

1032 Cell Based IL12 Immunomodulation Combined with PD-1 Inhibition Generates Local and Abscopal Immune Activation to Eradicate Metastatic Pancreatic Cancer

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Introduction: Proinflammatory Cytokines have been FDA approved for cancer immunotherapy for treatment of metastatic melanoma and renal carcinoma for over 30 years. To overcome stability and toxicity limitations seen with high dose cytokine immunotherapy, we developed a delivery platform, called cytokine factories, composed of genetically engineered epithelial cells encapsulated in biocompatible polymers. Cytokine factories can safely deliver high local doses of pro-inflammatory cytokines, such as interleukin- 2 (IL2) and 12 (IL12) and allow for controlled and programable dosing *in vivo*. The IL2 producing version of this technology has advanced to human clinical trials for the treatment of ovarian cancer. Here, we highlight our advancements towards further development of this technology to produce IL12 in combination with PD1 check point blockade to treat metastatic pancreatic cancer and metastatic models of melanoma. **Results:** Tumor-adjacent administration of IL12-based cytokine factories *in vivo* created a high local cytokine concentration (IP space) without substantial leakage into the systemic circulation. In addition, administration of cytokine factories in combination with anti-PD1 checkpoint inhibitors caused reduction of tumor burden by over 60% when delivered as a monotherapeutic to mice with metastatic melanoma. Further, when administered in combination with local anti-PD1 checkpoint inhibitors, these cytokine factories led to reduction of intraperitoneal tumor burden by over 80% after only 10 days of treatment. Finally, we evaluated the ability of this therapy to treat pancreatic tumors which are notoriously known to be resistant to immunotherapy. We found that the median survival for control animals was 45 days. However, 8/8 of animals treated with IL12 cytokine factories survived throughout the duration of the study (110 days), demonstrating a significant extension of overall survival time. Finally, the pharmacokinetics, pharmacodynamics and safety of this system were evaluated in non-human primates (NHP). A single administration of led to therapeutic levels of IL-12 in the intraperitoneal cavity in NHP and produced local and systemic T cell biomarker profiles that predict efficacy. In addition, administration of cytokine factories at various doses in NHP were well tolerated with no signs of cytokine storm and vascular leak syndrome with no abnormal clinical observations and no evidence of adverse pathologic effects on local or systemic tissue by all animals during the study. **Conclusions:** Our findings demonstrate efficacy of cytokine factories as single agent and combination therapeutics in preclinical animal models and provide rationale for future clinical testing for the treatment of metastatic peritoneal cancers in humans.

1033 MRI Guided Delivery to the Putamen Using the Northern Arc: A Custom Frame Allowing Occipital Approaches in Large Animal Models

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Introduction: Delivery of gene therapies directly to the central nervous system allows the blood brain barrier to be bypassed and decreases the risk for systemic exposure and toxicity. Intraparenchymal (IP) delivery directly to the brain using Convection Enhanced Delivery (CED) is ideal for maximizing exposure of the target of interest (e.g., putamen, caudate, substantia nigra) to the therapeutic agent. Currently, multiple targeting devices are used for CED in the human patient. However, application of devices scaled for a human can be challenging to apply to a large animal laboratory species. Northern Biomedical Research (NBR) has developed a custom Magnetic Resonance (MR) compatible system (the Northern ARC) to allow bilateral infusion device placement and for simultaneous, real-time MRI guided CED. The frame allows a full range of targeting, from anterior/frontal to occipital approaches. The objective of this study was to assess feasibility and effectiveness of the Northern ARC for infusions into the putamen using occipital trajectories. **Methods:** Three cynomolgus macaques were anesthetized and placed in a semi-prone position in the Northern ARC frame. Bilateral craniotomies were made for an occipital approach to the putamen and the animals were transferred to the MRI (Philips Achieva 3.0T). The putamen was targeted using gadolinium-filled arrays (2 mM ProHance, Bracco) attached to the frame. The cannulae (Clearpoint Neuro Smarflow Cannula) were primed with dosing solution containing 2 mM ProHance for infusion visualization and

marked at four depth locations. The cannulae were inserted to the proximal boundary of the target and advanced stepwise to optimize target coverage. Infusions commenced at 1 - 3 μL per minute. T1 scans were performed prior infusion and continuously during the infusions for distribution monitoring. The bilateral infusions were performed simultaneously. For all three animals, DICOM MR images were provided to Brainlab (Brainlab AG, Munich, Germany) for image post-processing and data analysis. For volumetric assessment a customized algorithm for auto-segmentation of the target structures and the infusate distribution by subtracting the baseline images from the post infusion images was applied. The infusate distribution volume overlapping the target structure was considered as coverage and is reported as a percentage of the target volume. **Results and Conclusion:** Six catheter placements were performed in three animals using the described procedure. Injection volumes of 85 - 125 μL per putamen, distributed over 4 injection sites, resulted in a coverage of 63 ± 3% of the putamen (maximum: 67%, minimum 58%). All catheters were placed on target. In conclusion, targeting the putamen for catheter placement by an occipital approach for simultaneous, bilateral CED infusions is feasible and effective in large animal species using the NBR Northern ARC Brain Infusion System. Analysis of infusate distribution through co-infused contrast agent showed high consistency in the level of target coverage.

1034 A High-Throughput AAV-Based Screening Platform for the Engineering of Cell-Type Specific Gene Regulatory Elements

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The fundamental cause of many neurological disorders can be ascribed to the abnormal electrical activity of specific neural circuits. Modifying the activity of neurons should therefore be a valuable avenue for the development of therapeutics to ultimately treat various nervous system conditions. However, for this to be a successful therapeutic approach, a high degree of specificity is required such that anomalous neuronal activity can be corrected in one circuit without altering the function of neighbouring cells and brain regions. To advance the treatment of neurological circuit disorders, Sania Therapeutics is leveraging a combination of intersectional technology platforms that allow for the targeted delivery of chemogenetic proteins through engineered adeno-associated virus (AAV) capsids and promoters. This approach will allow us to specifically modify neural circuit activity in disease states. Here we describe the development and validation of Gre-Scan, an AAV-based screening platform for the identification of cell type specific gene regulatory elements (GREs) such as promoters and enhancers. The use of cell type specific GREs is a promising way of targeting therapeutic genes to dysfunctional cell and tissue types. The ideal GRE for therapeutic purposes would drive high levels of gene expression in the targeted cell type, with minimal off target expression, whilst being compact enough to permit use within the limited packaging capacity of AAV vectors. Several fundamental challenges have so far precluded the identification and widespread use of cell type specific promoters, particularly in AAV-based genetic therapies. First, the relatively unmapped nature of gene regulatory elements within the genome has meant that identifying putative GREs has been challenging. Second, when potential GREs are identified there are few high throughput ways of measuring their activity in biological systems. Methylation-specific high throughput sequencing methods such as ATAC-Seq, combined with bioinformatics analysis, have vastly increased the number of putative GREs that could potentially drive cell type specific expression in AAV vectors. However, methods for the biological screening of these sequences have lagged behind and are generally restricted to laborious individual assessment, or tagging with genetic barcodes - the upper limit of which is generally in the 10s or 100s range per study. Here, we describe a biological screening platform that can be used for the high throughput screening of GRE activity across cell and tissue types. The screening process starts with the construction of large, diverse GRE libraries within AAV vectors. These libraries are built either through combinatorial expression of known transcription factor binding sites or putative GRE sequences identified by next generation sequencing. The Gre-Scan platform is then used to screen these libraries in parallel across multiple cell and tissue types. Sequences of interest identified by the platform can then be further validated on an individual basis. In a separate set of experiments we rationally designed a small library of GREs based on human motor neuron transcription factor binding sites and compared their activity in vitro individually and as part of the Gre-Scan platform. We have further validated this platform through the investigation of GREs that drive gene expression selectively in human motor neurons. We have built a human motor neuron specific GRE library and used the GRE-Scan platform to identify sequences that specifically drive gene expression in those neurons. This platform has led to the identification of the most promising candidates to take forward for in vivo testing for use in targeted therapeutics.

1036 EG-70, a Novel Non-Viral Gene Therapy Inducing Transgene Expression with Anti-Tumor Activity in the Peritoneal Cavity

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Background: enGene is developing a novel non-viral vector for gene therapy. The vector platform consists of a proprietary polymer, arginine-glucose derivatized-oligomeric chitosan (DDX), complexed with plasmid to form polydispersed nanoparticles with an average diameter of ~100nm. The core nanoparticle is further coated with an anionic methoxy-poly(ethylene glycol)-poly(L-glutamic acid) block copolymer. This delivery vehicle is stable in mucus and exhibits exceptional muco-penetration properties. EG-70 (detalimogene voraplasmid) is a nanoparticle formulation based on this DDX platform comprising a plasmid that expresses two non-coding RNA products that agonize the innate immune receptor retinoic acid-inducible gene I (RIG-I), as well as a single-chain recombinant human interleukin-12 (IL-12). This immunotherapy, with multiple mechanisms of action, has the potential to remodel the tumor microenvironment and bridge the innate and adaptive anti-tumor immune responses. In murine preclinical studies, EG-70 was found to be safe with minimal

and reversible local toxicity, to result in transfection of bladder urothelium, to produce the transgene products, and to drive a durable tumor-specific immune response in an orthotopic model of bladder cancer. EG-70 is being assessed in a Phase 1/2 clinical trial (LEGEND, NCT04752722) to evaluate its safety and efficacy in patients with Bacillus Calmette-Guerin (BCG)-unresponsive non-muscle invasive bladder cancer (NMIBC) and has shown promising preliminary outcomes in a first-in-human clinical study (NCT04752722). EG-70's formulation and anti-tumor mechanism are both broadly applicable for malignancies where there is unmet medical need. Patients afflicted with ovarian cancer are commonly diagnosed at an advanced stage and receive a poor prognosis. This study aims to evaluate the potential clinical benefit of EG-70 with intraperitoneal administration for ovarian cancer. **Results:** Intraperitoneal administration of a species-specific surrogate drug product (nanoparticles containing a plasmid DNA encoding for the mouse IL-12 homolog, along with the two RNA products) is well tolerated in mice. Local IL-12 protein expression, as assessed by MSD, is robust and reversible in the peritoneal lavage fluid. Dose-response studies were conducted to determine the optimal dose to drive high levels of expression, which is dependent on several factors including the volume of administration, the dose, and the concentration. Dose-dependent activation of downstream pharmacodynamic effects, such as IFNγ production, was observed. Administration of mEG-70 in a syngeneic murine model of ovarian cancer resulted in a significant reduction of tumor burden, compared to control animals. Further analyses are underway to characterize the remodeling of the tumor microenvironment following administration of EG-70. **Conclusion:** These results demonstrate that the novel DDX non-viral gene therapy platform can efficiently and safely deliver gene therapy to the peritoneal cavity for local anti-tumor transgene expression and drive a dose-dependent pharmacodynamic response. The level of transgene expression is biologically significant and supports the continued development of EG-70 for ovarian cancer or other malignancies within the peritoneal cavity.

1037 Development of Effective and Safe New Gene Therapy Drug (ARU-2801) for Hypophosphatasia

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Hypophosphatasia (HPP) is an inherited bone disease caused by a deficiency of tissue-nonspecific alkaline phosphatase (TNAP). Enzyme replacement therapy requires subcutaneous injection 3-6 times a week for the rest of the patient's life. We evaluated the efficacy and safety of a gene therapy drug (TNAP-expressing type 8 adeno-associated virus vector; ARU-2801) to develop an alternative treatment. ARU-2801 [$1.0x10^{11}$, $3.0x10^{11}$, $1.0x10^{12}$ vector genomes(vg)/body] was injected intramuscularly once during the neonatal period (P1-3) to *Alpl* $^{-/-}$ mice, and survival, improvement of bone formation and plasma alkaline phosphatase (ALP) activity were investigated for 18 months. In Non-human primates (NHPs), ARU-2801 was injected once intramuscularly at 1.0 $x10^{13}$ vg/body and evaluated the safety for up to 84 days. All untreated *Alpl* $^{-/-}$ mice (n=4) died within 1 month. Over $3.0x10^{11}$ vg treated *Alpl* $^{-/-}$ mice (n=14) maintained high plasma ALP activity around 10,000 U/L and showed prolonged survival. *Alpl* $^{-/-}$ mice in the $1.0x10^{12}$ vg group (n=7) exhibited weight gain, and bone mineral density as well as wild-type mice. The treated *Alpl* $^{-/-}$ mice showed no abnormal blood chemistry tests, and no tumor formation or ectopic calcification. The treated NHPs also showed sustained plasma ALP activity with no toxicities. Thus, ARU-2801 was effective and safe in both *Alpl* $^{-/-}$ mice and NHPs. ARU-2801 could be a potentially life-prolonging and quality-of-life-improving agent for HPP patients that warrants further development as a novel treatment alternative to current enzyme replacement therapy.

1038 Positive Results from a First-in-Human Study Support Continued Development of PGNEDO51 for the Treatment of Duchenne Muscular Dystrophy (DMD)

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Background: PepGen's Enhanced Delivery Oligonucleotide (EDO) cell-penetrating peptide technology is engineered to optimize tissue delivery and cellular uptake of therapeutic oligonucleotides. Delivery of oligonucleotides to affected tissues is a major challenge that limits their efficacy. PGN-EDO51 is being evaluated for the treatment of DMD amenable to exon 51 skipping. **Objectives:** To evaluate the safety, tolerability, pharmacokinetics (plasma and muscle), and pharmacodynamics (exon skipping) of single-ascending doses of PGN-EDO51 administered intravenously (IV) to healthy male volunteers (HV). **Methods:** Thirty-two adult HVs were randomized to receive a single dose of PGN-EDO51 (1, 5, 10, or 15 mg/kg; n=6 per cohort) or placebo (n=2 per cohort). Safety and tolerability were monitored over 28 days. Biceps needle biopsies were performed on Days 10 and 28 for measurement of tissue concentrations of PGN-EDO51 and exon skipping (pharmacodynamic biomarker to demonstrate target engagement). **Results:** All HVs completed the study. The majority of treatment-emergent adverse events were mild and resolved without intervention, including transient, reversible changes in kidney biomarkers (n=9) and hypomagnesemia (n=2) at the highest doses, with no significant clinical sequelae. At Days 10 and 28, respectively, there were dose-dependent and sustained concentrations of PGN-EDO51 measured in biceps biopsies: 9.7 nM and 3.8 nM (5 mg/kg); 19 nM and 11 nM (10 mg/kg); and 50 nM (both days at 15 mg/kg); and dose-dependent increases in mean exon skipping of 0.14% and 0.35% (5 mg/kg); 1.1% and 1.4% (10 mg/kg); and 1.4% and 2.0% (15 mg/kg). **Conclusions:** The Phase 1 study results demonstrate that PGN-EDO51 has a generally tolerable profile at clinically relevant doses. PGN-EDO51 exhibited high levels of muscle oligonucleotide delivery and

exon 51 skipping following a single dose. PGN-EDO51 concentrations and levels of exon skipped transcripts in muscle at Day 28 indicate the potential for accumulation of exon 51 skipped transcripts and dystrophin protein with repeat dosing in people with DMD amenable to exon 51 skipping. Based on these results, we plan to initiate a Phase 2, multiple-ascending dose study in people with DMD in the first half of 2023.

1039 Kinetics of Innate and Adaptive Immune Responses to Adeno-Associated Virus in a Preclinical Model

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Adeno-associated virus (AAV) vectors are leading candidates for gene therapy product delivery. However, immune responses to AAV or transgene products represent major hurdles to widespread clinical use. AAV induces long-lived T and B cell responses that have the potential to eliminate transgene expressing cells and prevent re-dosing. Thus, characterizing the anti-vector innate activation, adaptive responses and their influence on transgene longevity is of great interest. AAVrh32.33 is a highly immunogenic capsid that induces robust T and B cell responses in mice making it a useful preclinical model. Innate immune activation triggered by the vector facilitates antigen presentation and priming of B and T cell responses (Mays et al. 2009, Faust et al., 2013, Mays et al., 2014). In this study, we investigated the kinetics of innate and AAV-adaptive responses and long-term transgene expression following intramuscular (IM) delivery of AAVrh32.33. We assigned groups (n=4) of adult female B6(Cg)-Tyrc-2J/J albino mice to receive AAVrh32.33 carrying a luciferase reporter (AAVrh32.33-CBA-Luc) or mock vehicle by IM injection into the right tibialis anterior. IVIS imaging monitored luciferase transgene expression in life and at sacrifice. Mice were sequentially sacrificed at 2, 5, 8, 14, 24, 72 hours or on days 7, 30 or 98 post-dose. Resiquimod (R848), a TLR7/8 agonist, was used as a positive control for innate stimulation and given to mice at 100 μg dose by IV/IM injection. Immune gene expression in the muscle was assessed by quantitative PCR of mRNA extracts and reported as relative fold change over mock-treated animals. An upregulation of select innate genes including interferon gamma induced protein 10 (IP-10) was observed beginning at 24 hours post dosing (~4 fold). In contrast, R848 induced robust expression of IP-10 2-5hr post dose (100-350-fold). AAV specific antibodies were measured by ELISA and T cell responses were assessed by IFN-γ ELISPOT and multi-parameter flow cytometry staining. By day 30, AAVrh32.33 induced high titer antibodies and robust T cell responses measured in the spleen (ELISPOT ≥1500 spot forming units per million). Additionally, antigen experienced AAVrh32.33pMHCI dextramer positive cells were present in spleen, liver, and bone-marrow (1-5% AAV dextramer$^+$ CD8 T cells). Gene expression analysis in the muscle indicated T cell infiltration as CD4 (51-fold), CD8 (174-fold), IFN-γ (79-fold) and IL-12 (433-fold) all increased in AAV treated animals relative to mock controls. Antibody and T cell responses contracted over the following 2 months but were still detectable at day 98. Luciferase transgene expression was detected in the muscle of AAV transduced mice by 8h and draining (popliteal and inguinal) lymph nodes by 24h. Transgene expression increased in magnitude during the first 14 days post AAV dosing, plateaued, and persisted at high levels for the duration of the study. Overall, the development of functional AAVrh32.33-specific T cell responses and T cell infiltration within the muscle did not ablate transgene expression. Thus, the persistence or loss of transgene is not solely dependent on the immunogenicity of the capsid used.

1040 5′ UTR Design for High Protein Expression Efficiency of mRNA Therapeutics/ Vaccines

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The 5′ untranslated region (5′ UTR) of mRNA is a crucial element in determining the translation efficiency of mRNA therapeutics/ vaccines. Therefore, understanding the 5′ UTR sequences that induce high ribosome loading efficiency is important for designing mRNA therapeutics/vaccines exhibiting high protein expression efficacy. In this regard, there have been various attempts to develop algorithms to predict the ribosome loading efficiency of 5′ UTR sequences and generate artificial 5′ UTR sequences expected to show high translation efficiency. The algorithms have been developed primarily based on the massively parallel reporter assays (MPRAs) of the 5′ UTR random library. However, the dataset of the current approaches inevitably contains a non-randomized additional sequence for primer binding at the 5′ ends of the 5′ UTR, which limits the exploration of the effect of the 5′ ends of the 5′ UTR library. To overcome this, we prepared a fully randomized 5′ UTR library without introducing additional sequences. Using this, we sought to dissect the role of 5′ ends of the 5′ UTR in determining ribosome loading and translation efficiency of mRNAs. Also, we tried to develop a 5′ UTR design algorithm to achieve high protein expression efficiency of mRNA therapeutics/vaccines.

1041 Amino Acid Analysis Indicates Metabolic Differences in Multi-Cytokine Backpack -Manufactured CAR T-Cells

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Novel reagents that aid in the consistent manufacturing of CAR T-cells with well-defined phenotypes are needed to improve treatment outcomes in patients. Traditional *ex vivo* expansion reagents provide only TCR activation and co-stimulation. Recently developed multi-cytokine backpacks (MCBs, Ref 1) have been shown to provide CAR T-cell manufacturing with additional cytokine support, critical for cell expansion, differentiation, and the selection of potent, long-lived phenotypes. A commonly used method for *ex vivo* activation is using the cytokine IL-2 with Dynabeads™. In this project (Ref 1) different

combinations of immunomodulating proteins were used to create an MCB library. The activated cells were tested for viability, CAR expression, phenotype and *in vivo* activity (Ref 1). Here we describe the amino acid (AA) analysis done with the REBEL device (908 Devices) to assess differences in the metabolic activity in these activated cells. The differences in metabolism were measured for all groups with Dynabead + exogenous IL-2 as the control and a reference point. **Methods** The T-cells were isolated and transduced as described in Ref 1. after activation, the cells were expanded in RPMI-1640 + 10 % FBS in a total volume of 200µL. AA concentrations were measured from the fresh and spent (end-point) media using the REBEL (908 Devices Inc.), a capillary-electrophoresis (CE) - mass spectrometry (MS) -based device for AA analysis. CAR T-cell products were isolated via centrifugation / 400xg for 5 minutes, and the cell-free supernatant was diluted 1:10 with manufacturer-provided diluent. Automated quantitation of AAs for each sample was achieved using embedded calibrations. Two biological replicates of each sample were analyzed in triplicate using the REBEL. The nutrient compositions of the final CAR T-cell product spent media were analyzed to identify differences in their cellular metabolism. The ratio of the mean AA concentrations from the microparticle backpack library samples to the mean concentrations from the control samples (Dynabead + IL-2) were plotted using 908 Devices add-in for JMP software. **Results** Based on several biological assays, the MCB library particles [UB3] were categorized as "high", "intermediate", and "low" activity with respect to CD8+ and CD4+ less-differentiated CAR T-cell phenotypes (T_{CM} and T_{SCM}). A focus on the top candidates (#09, #16, #27, and #43) spent media samples interestingly showed differences as compared to control in concentration for the AAs Ala, Gln, Gly, Pro, Ser, Thr. It was observed that some AAs (ie. Ala, Gly, Ser) were accumulating in the media during culture in all samples, whereas some (Arg, Ser) were consumed. **Conclusions** AA analysis is not regularly performed as part of CAR T-cell experiments due to the complexity of many traditional analysis methods and sample volume limitations. With automated quantitation and low sample volume requirements, the REBEL device enables rapid analysis at the bench side with no requirement for prior MS expertise. Studies to define relationships between AA dynamics, T-cell metabolism, and CAR T-cell biology are currently in progress; however previous findings by Ref 2 indicate that AAs are critical for efficient T-cell activation and proliferative responses. With this work we demonstrate a correlation between CAR T-cell activation, nutrient metabolism, and subsequent therapeutic efficacy. Ref 1 - Lin and Uricoli et al. 2023 Nature Biomedical Engineering; Submitted Ref 2 - Cobbold SP, *et al*. Proc Natl Acad Sci U S A (2009) 106:12055-60

1042 Development of an AAV-Based microRNA Gene Therapy for Myotonic Dystrophy Type 1

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Myotonic Dystrophy Type 1 (DM1) is the most common form of adult muscular dystrophy (~1:8000) characterized by life-threatening muscle weakness, compromised respiration and often cardiac conduction abnormalities with high unmet medical need. DM1 is caused by a CTG repeat expansion in the 3' untranslated region (UTR) of the dystrophia myotonica protein kinase (DMPK) gene, resulting in DMPK mRNA hairpin structures that aggregate as insoluble ribonuclear foci and sequester several RNA-binding proteins. The resulting redistribution of essential splicing factors, such as muscleblind-like 1 (MBNL1), causes mis-splicing of downstream effectors responsible for the differentiation of muscle tissue. Targeting DMPK RNA appears to hold some of the greatest prospects for therapeutic intervention in DM1. Gene therapies with siRNA or ASOs against DMPK mRNA have shown promise in animal models and in clinical trials, but require repeated administrations. To overcome this limitation, we developed an artificial miRNA to target human DMPK mRNA for constitutive expression following systemic delivery of a recombinant adeno-associated vector (rAAV). To determine the potential of amiRDMPK to correct the DM1 phenotype, we first tested their ability to silence the human DMPK transcript and correct splicing defects in DM1 patient cells. The treatment of those cells with amiRDMPK caused over 50% DMPK mRNA silencing and splicing defect correction measured by MBNL1 exon 7 inclusion. Next, we evaluated the same approach in the DMSXL mice, a DM1 mouse model carrying a human *DMPK* gene with toxic CTG repeats. Our results show that a single intravenous injection of the AAV vector expressing the amiRDMPK, resulted in significant reduction of the toxic human nuclear DMPK mRNA in muscle, including the heart. Consequently, a reduction in the number of nuclear foci and reversion of splicing abnormalities were observed. Furthermore, treatment with amiRDMPK improved body weight, survival rate, cardiac and skeletal muscle function. Lastly, we tested our approach in non-human primates (NHP), testing three different doses. In this study, we found a significant, dose-dependent accumulation of AAV genomes in all the relevant tissue (namely skeletal and cardiac muscle). This resulted in dose-dependent reduction of DMPK expression, up to 90%, as compared to a control group. Importantly, all doses tested in NHPs resulted to be safe and well-tolerated. Overall, our data provide evidence on the efficacy of artificial miRNAs against *DMPK* as a promising AAV-based gene therapy for DM1.

1043 RNA Reprogramming Therapeutics Based on Tetrahymena Group I Intron for Hepatocellular Carcinoma

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Group I intron-based trans-splicing ribozyme enables to sense and reprogram target RNA into gene of interest through RNA replacement. Previously, we proposed hTERT-targeting trans-splicing ribozyme harboring therapeutic suicide gene for cancer therapy. Hepatocellular carcinoma (HCC) is major type of primary liver cancer, and its incidence and mortality rates are steadily increasing. However, HCC has high fatality rate and limited therapeutic options. Recently, Immune checkpoint inhibitor for activating the function of T cells is the fastest growing therapeutic option in the cancer therapy, but immunotherapy has low response rate and limitation to applying it to all cancer patients. Here, we optimized the specific ribozyme for highly efficient

anticancer activity with less off-target effect for anti-HCC approach. To confer potent anti-HCC effects and minimize hepatotoxicity, we constructed post-transcriptionally enhanced ribozyme expression cassettes harnessed with splicing donor, acceptor site, and woodchuck hepatitis virus post-transcriptional regulatory element under the control of microRNA-122a, the expression of which is down-regulated in most HCC. Then, systemic or local administration of adenovirus encoding our refined ribozyme, termed RZ-001, achieved great anti-cancer efficacy and improved ability to specifically target cancer without hepatotoxicity in both nude and humanized xenograft mice model. Of note, the immune cells were found to be effectively infiltrated into cancer tissue after RZ-001 administration in the humanized xenograft mice model. Through GLP toxicity and biodistribution study, minimal liver toxicity and tissue distribution and clearance pattern of the recombinant adenovirus were observed in normal animals administered systemically. Based on these results, RZ-001 recently received IND approval for first-in-human clinical 1/2a trials of group I intron based trans-splicing ribozyme from the Korean Ministry of Food and Drug Safety and the United States FDA, and was administered to the first HCC patient. Reprogramming of hTERT mRNA by post-transcriptionally regulated RNA replacement strategy mediated by a cancer-specific ribozyme would provide a clinically relevant, safe, and efficient strategy for HCC treatment.

1044 Biodistribution Assessment in Non-Human Primates of JAG201, a *SHANK3* AAV9 Vector Delivered via ICV Injection for ASD, Phelan-McDermid Syndrome, and Other SHANK3 Mutation or Deletion Related Conditions

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Background: The SH3- and ankyrin repeat (SHANK) protein family are major scaffolds of the post-synaptic density of excitatory synapses, where loss of *SHANK3* function provokes synaptic dysfunction in the brain. Affected patients present with a debilitating neurodevelopmental disorder characterized by severe intellectual disabilities, impaired or absent speech and language, deficits in motor skills, behavioral challenges, and autism and/or autistic features. JAG201 is as an investigational AAV9-based gene therapy in preclinical development intended to deliver a functional version of *SHANK3* to treat autism spectrum disorder (ASD), Phelan-McDermid syndrome (PMS), and other neurodevelopmental disorders that result from a mutation or deletion within the gene or from chromosomal rearrangements at 22q13.3 spanning *SHANK3*. Genetic sequencing studies indicate that *SHANK3* mutations or deletions may be present in ~1% of patients with ASD, equating to about 30,000 patients in the U.S. Here, we present data from a non-GLP study in NHPs evaluating the biodistribution of vector genome DNA and RNA transgene expression of JAG201 in the CNS and peripheral organs following unilateral and bilateral intracerebroventricular (ICV) administration. ***Methods:*** 24 NHPs (12 males and 12 females) aged approximately 2-3 years received either a single ICV administration of vehicle or JAG201 via unilateral or bilateral injection at a dose of 1.0E13 or 1.0E14 vg/animal in a total volume of 2.0 mL given via bolus injection. Injection coordinates were determined via MRI followed by stereotaxic administration targeting the lateral ventricles. Animals were followed for a 90-day in-life period, and biodistribution of JAG201 vector DNA and RNA in CNS and peripheral tissues were analyzed via droplet digital PCR (ddPCR) and supported via RNA fluorescence *in situ* hybridization (FISH). ***Results:*** The unilateral and bilateral ICV administration procedures and single doses of JAG201 were well tolerated. At 90 days, animals that received JAG201 showed widespread rostrocaudal transduction throughout the CNS in a dose-dependent manner compared to vehicle controls. Analysis of JAG201 vector genome DNA copies in five key brain regions showed the highest levels of transduction in the frontal cortex and hippocampus, followed by the cerebellum and spinal cord, at both dose levels. Widespread transduction of the striatum and thalamus was observed at the higher JAG201 dose level. These findings were supported and mirrored by JAG201 RNA quantification and RNA FISH analysis, with dose-dependent expression observed in all evaluated brain and spinal cord regions. Additionally, unilateral and bilateral JAG201 administration resulted in comparable overall levels of vector DNA and RNA copies throughout the brain, including comparable levels in both the contralateral and ipsilateral sides of the brain. Analysis of vector DNA copies in peripheral tissues confirmed leakage of vector from the CNS, however in contrast to the CNS, significantly lower relative levels of RNA expression were observed as a result of the highly neuron-specific activity of the human synapsin promoter limiting off-target transgene expression. ***Conclusion:*** These data provide evidence that both unilateral and bilateral ICV administration of JAG201 result in comparable widespread biodistribution throughout the CNS. Further, JAG201 RNA expression analysis confirms widespread expression throughout the brain and spinal cord while limiting off-target expression in peripheral tissues. In summary, these findings confirm the selection of unilateral ICV administration for further evaluation of JAG201, a *SHANK3* AAV9-based gene therapy targeting *SHANK3* mutation or deletion related disorders.

1045 Senescent Cells Accumulation During Human Muscle-Derived Stem Cells Expansion Negatively Affects BMP2-Mediated Bone Regeneration

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Introduction. It has been shown that human muscle-derived stem cells (hMDSCs) can promote bone regeneration in a critical sized calvarial bone defect when transduced with lenti-viral BMP2. Coacervate is a polymer that can sustained-release growth factors for tissue engineering applications. The aim of this study is to use the coacervate sustained release platform to investigate whether coacervate delivered BMP2 can promote bone formation and if adding human muscle-derived stem cells is beneficial for bone regeneration mediated by BMP2-coacervate.

Methods. **1.** Synthesis of poly (ethylene argininylaspartate diglyceride) (PEAD) and heparin coacervate was performed as previously reported. **2. hMDSCs** were isolated with a modified pre-plate technique and transduced with lenti-GFP. **3. *In vivo* bone regeneration using critical sized calvarial bone defect model**: male ISCRSCID mice were divided into 2 groups (n=6): group 1: 2 μg BMP2+coacervate+1.5×10^6 hMDSCs/LGFP; group2: 2 μg BMP2+coacervate. Fibrin sealant was used as a scaffold. Critical sized 5mm calvarial bone defects were created on the right parietal bone of mice. For the BMP2+coacervate+hMDSCs group, the BMP2 coacervate was first mixed with hMDSCs (20ul in PBS) and then added 20ul thrombin, mixed, added to the defect, then 20ul fibrinogen was added to the defect, and allowed to form a fibrin gel. For the BMP2+ coacervate group, BMP2 coacervate was mixed with 20ul thrombin, added to the defect, and then 20ul fibrinogen was added. **4. MicroCT and histology**. Bone regeneration was quantified using the Viva-CT 80 at days 1, 14, 28 and 42 lively. Mice were sacrificed at 6 weeks after surgery and skulls were harvested and fixed in formalin for histology. H&E, Herovici's staining was performed to reveal general morphology and bone matrix collagen 1 (Col1). **5. Cellular senescent detection**. hMDSCs/LGFP at passage 6 and 10 after lenti-GFP transduction were seeded in 24 well plate and cultured for 48hrs. 3 wells of each passage were used for senescent associated-β-gal staining, while 3 wells from each passage were used for total RNA extraction, Q-PCR for P16INK4a and interleukin 1β. **Results.** MicroCT results showed limited new bone formation in the BMP2+coacervate+hMDSCs group while the BMP2 coacervate group (without cells) showed robust new bone formation. BMP2+coacervate group regenerated significantly more new bone than the BMP2+ coacervate+hMDSCs/LGFP group at all time points. H&E staining showed small island-like areas of bone in the defect area in the BMP2+coacervate+hMDSCs group. In contrast, functional new bone with bone matrix and bone marrow was formed in the BMP2 coacervate group**.** Herovici's staining showed nearly no new bone was formed in the middle of the defect of the BMP2+coacervate+hMDSCs/LGFP group. However, half of the defects were filled Col1 positive new bone in the BMP2+coacervate group. These unexpected results further prompted us to investigate why adding hMDSCs decreased new bone formation mediated by BMP2 coacervate. We found that more SA-β Gal positive cells are present at passage 10 (the cell passage we used in vivo for this study) when compared to passage 6. The P16 and IL1β also increased in passage 10 compared to passage 6. **Conclusion.** Our study revealed that the use of coacervate to deliver BMP2 regenerated new bone that are comparable to Lenti-BMP2 transduced hMDSCs as reported previously. However, to our surprise, adding later passage of hMDSCs to BMP2 coacervate significantly decreased new bone formation despite using same dose of BMP2. This adverse effect was likely due to accumulation of senescent cells at advanced passage.

1046 Separation of Empty, Partial, and Full Capsids Using Multicolumn Chromatography

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AAV capsids that carry and deliver the desired transgene to the target cells are one of the most popular viral gene transfer vectors. In addition to capsids carrying the desired transgene, AAV production often results in many capsids devoid of the transgene (empty capsids) or containing a truncated transgene (partial). Before downstream purification, these capsid variants are often present in a larger percentage than the full capsids and can potentially reduce the vector's effective dosage. Product consistency also necessitates proper control of the product profile in the final drug substance, calling for a scalable purification method that can separate these product variants. Furthermore, the enrichment and separation of these variants can enable the proper characterization of each variant and the investigation of its biological impact. Anion exchange chromatography (AEX) is widely used to separate empty and full capsids. However, the enrichment and separation of partial capsids with anion exchange chromatography have not been demonstrated in the industry. Partial AAV capsids are heterogeneous and have features that overlap with full capsids, making it difficult to separate them from full AAV capsids. Typically, a shallow salt gradient elution separates empty and full capsids. Often, sufficient resolution between empty and full capsids cannot be obtained, and the reliance on salt gradient elution often leads to lower product yield. In this work, empty, partial, and full capsids are enriched and separated by self-displacement chromatography, wherein variants with a stronger affinity to the AEX resin displace the variants with a weaker affinity to the resin. We demonstrate unparalleled separation of empty, partial, and full capsids with single- and multicolumn anion exchange displacement chromatography. The approach employed here will help to control product variants and, in general, aid in their isolation to enable further characterization.

1047 MyoSite Human Skeletal Muscle-Derived Cells (skMDC) as a Tool for Studying Neuromuscular Disorders

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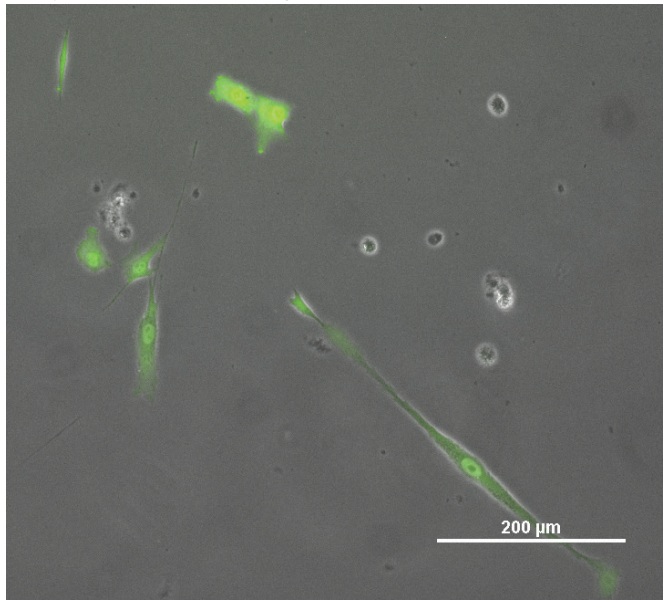
Cultured myoblasts are a valuable tool in cell biology for studying underlying mechanisms of neuromuscular and metabolic disorders, including muscular dystrophies and Type II diabetes. Immortalized cell lines, such as C2C12 myoblasts have played a pivotal role in the research of in vitro research in neuromuscular diseases. However, these cells are mouse derived and may not accurately model neuromuscular processes in humans. Primary human skeletal muscle derived cells (skMDCs) are proliferative myoblasts that are capable of differentiation into multi-nucleated myotubes. skMDCs can be isolated from both healthy donors, as well as donors with inherited mutations such as those implicated in muscular dystrophies. Gene editing in both C2C12 cells and primary myoblasts has shown to be challenging due to difficulties in transfection. Lipid-mediated transfection by commercially available reagents while highly efficient in various immortalized cell lines, can be more challenging in primary cells. Electroporation is an alternative method to introduce plasmids and ribonucleoprotein into cells through transiently increasing the permeability of the cell membrane by application of a short electric pulse. One major drawback to electroporation-mediated transfection is cell death during the pulse, thus requiring optimization. In the present study, we evaluated the transfection efficiency of an enhanced green fluorescent protein (eGFP) containing plasmid using the Bio-Rad GenePulser XCell™ system in MyoSite skMDCs. skMDCs were cultured in MyoTonic™ Basal Media supplemented

with MyoTonic™ Growth supplement, 100 U/mL penicillin, and 100 µg/mL streptomycin, and cultures were maintained at 37 °C with 5% CO2. Prior to electroporation, cells were trypsinized and resuspended in complete MyoTonic™ Media without antibiotics to a concentration of 100k cells/100 µL. GFP containing plasmid was added to the cell suspension at 10 µg/mL and the cell were pulsed at 170V, 975 µF in 2 mm cuvettes using an exponential decay protocol. Cells were then diluted in complete media and plated in 6-well plates. eGFP expression was assessed 48 hours after electroporation by fluorescence microscopy. Analysis was performed using NIS-Elements BR (Nikon).



Transfection of eGFP by this approach with electroporation resulted in a transfection efficiency greater than 50% as determined by the number of GFP positive cells. Our results using eGFP as a reporter for transfection efficiency suggest that MyoSite skMDCs may serve as a cellular model requiring plasmid transfection or introduction of gene-editing materials in studies exploring molecular mechanisms of muscle cell development and neuromuscular disease.

1048 Multi-Gene Co-Transfection is Dependent on Transfection Method

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Delivery of multiple genes to a single cell is necessary for many research and therapeutic purposes, including generation of iPSCs, viral reverse genetics, and molecular pathway analyses. However, as increasing numbers of distinct genes are delivered, the efficiency of every distinct gene being delivered to a given cell is reduced. This creates an expensive and frustrating bottleneck in multigene studies and may complicate analysis when some cells only receive part of the total desired genetic cargo. Various non-viral transfection methods have been used to co-deliver multiple genetic cargoes, most commonly lipofection and electroporation. We analyzed co-transfection of both methods through their ability to deliver plasmids expressing different fluorescent proteins. We further evaluated how increasing the amount of plasmid used in the transfection affected co-transfection and cytotoxicity. Co-transfection was analyzed using spectral flow cytometry and fluorescence microscopy, and cytotoxicity was analyzed using an LDH assay. By transfecting A549 cells we found that increasing the number of distinct plasmids being delivered resulted in fewer co-transfected cells, regardless of method. Lipofection resulted in greater co-transfection of A549 cells relative to electroporation. However, when using lipofection, increasing the number of distinct plasmids or increasing the quantity of plasmids delivered resulted in an increased cytotoxicity. Electroporation did not show this increased cytotoxicity with increased number of plasmids or quantity of plasmids, despite a higher baseline cytotoxicity. Increasing the amount of plasmid being delivered by electroporation also improved co-transfection. These data indicate that (1) the choice of transfection agent affects co-transfection and (2) increasing plasmid quantity in electroporation improves co-transfection.

1049 New 4th Generation Lentiviral Vectors with Simplified Vector Genome RNA Biosynthesis: How to Inactivate the Major Splice Donor Site and Retain High Titre

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To advance the utility of Lentiviral vectors (LVVs) it will be necessary to further improve the quantity and quality of vectors produced. The HIV-1 genome is structurally complex, with *cis*-acting elements including the major splice donor (MSD), multiple splice acceptor (SA) sites, packaging signal (Psi), and Rev-response element (RRE) governing production of unspliced and spliced RNA. Standard 3rd generation LVVs retain minimal HIV-1 sequences in their vector genomic RNA (vRNA) including Psi and RRE, in such a configuration that theoretically splicing activity is suppressed by Rev, leading to production of unspliced cytoplasmic vRNA for packaging. However, in reality up to 95% of vRNA transcripts may be spliced from the MSD to strong or cryptic SAs located within the transgene expression cassette, even when Rev is supplied during LVV production. Whilst only the unspliced vRNA contains a full Psi sequence, these aberrantly spliced mRNAs can lead to transgene protein production during manufacture, which is undesirable in the final vector product, even when tissue-specific promoters or other methods such as the TRiP System™ are employed to suppress expression (doi: 10.1038/ncomms14834). Addressing this issue through mutating the MSD is challenging due to its location within Psi. An intact MSD has also been shown to suppress the HIV-1 5' R polyadenylation site through recruitment of U1 snRNA - a redundant function shared with the auxiliary protein Tat. Accordingly, mutation of the MSD leads to a substantial decrease in LVV titres, and renders them Tat-dependent, which is clinically undesirable. We report on a new class of MSD-inactivated LVV genomes that do not aberrantly splice, leading to simplified production of a greater proportion of full length, packageable vRNA. These genomes are therefore also preferential when used in conjunction with the TRiP System™. An optimal MSD mutant was identified that minimises the attenuating effect on titres, and avoids activation of local cryptic

splice donor sites. Critically, Tat-independent production of these MSD-inactivated LVVs can be realised by two approaches. In the first approach, used for LVVs retaining the RRE so that transgene cassette introns are retained, a modified U1 snRNA molecule targeted to the Psi RNA region is co-expressed during production. In contrast to reported U1 snRNA functions, modified U1 snRNA was found to rescue titres independently of 5' polyA suppression. Through optimisation U1 snRNA binding length and target, MSD-inactivated LVV titres could be fully rescued to those of MSD-intact LVVs. Surprisingly, we also observed that modified U1 snRNAs were often found to improve the titres of 3rd generation (MSD-intact), clinical LVVs by 2-to-7 fold. The second approach is to use an MSD-inactivated LVV genome with a synthetic intron ('Vector-Intron' [VI]) in place of the RRE, thus making such LVV genomes Rev-independent. Splicing-out of the VI stabilises the vRNA, and therefore use of the modified U1 is not required. Together with additional deletions within the Psi-gag region, this liberates ~1kb of additional space. These 'MaxPax' LVVs cannot retain transgene cassette introns, and are therefore being evaluated for very large inserts where transgene space is limited, and introns are not desirable. Finally, these novel genomes, in combination with the TRiP System™, are being evaluated for use in generating stable producer cell lines for CAR-LVVs, where efficient suppression of the CAR protein appears to be correlative with success. The MSD-inactivated feature forms a core aspect of our 4th generation LVVs, and will be an attractive platform for future LVV product development in general.

1050 Superior Cytotoxic Signatures of Cord Blood Stem Cell-Derived Natural Killer Cell Therapeutics Identified with Bulk and Single-Cell Transcriptomics Analysis

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Glycostem Therapeutics has developed a closed, automated, and feeder-free system (uniK™) for *ex vivo* expansion and differentiation of umbilical cord blood-derived $CD34^+$ stem cells into highly functional, cryopreserved, truly off-the-shelf GTA002 NK cells, currently evaluated in a Phase I/II clinical study in AML, WiNK (NCT04632316). GTA002 batches are each generated from a different stem cells donor, however show similar patterns of expression of cell surface receptors and potent *in vitro* cytotoxicity against a variety of tumor cell lines. Nevertheless, the interindividual variability between donors may result in heterogeneity of functionality, which could not be predicted beforehand. Therefore, we investigated the transcriptome profile of multiple GTA002 batches, comparing the bulk (population level) and single cell (sub-population level) RNA-sequencing profiles. We actively selected batches with biological heterogeneity by comparing their *in vitro* cytotoxic potential against various cancer cell lines, evaluated under challenging low E:T ratio conditions. First, we selected 10 GTA002 pre-clinical batches, for which we monitored relevant biological parameters as surface receptor profile and potency, to distinguish a group of superior killers (4/10), compared to the rest (6/10). Next, we used bulk RNA-seq to investigate gene expression differences between the two groups. Interestingly, we found distinct, similar, transcriptional patterns for superior killers, and determined gene expression signatures via gene set enrichment and pathway analysis. In parallel, we pursued in-depth investigation of batch sub-populations by performing topological and clustering analysis via scRNA-Seq, also including multiple batches produced under good manufacturing practice (GMP) conditions. In depth-transcriptomics analysis of GMP batches is pivotal to advance our product understanding towards standardized off-the-shelf, allogeneic, NK cell therapies. Identification of biomarkers of excellent batches can then be linked to therapeutic efficacy, to provide the most appropriate product to a given patient. This research contributes to the development of universal cell therapies by generating a deep understanding of underlying donor- or process-induced variability, which is highly relevant and applicable to products originating from different sources, like induced pluripotent stem cells-derived NK cells (iPSC-NK).

1051 Establishing Product Quality Standards Early Streamlines Regulatory Filing for AAV Gene Therapy Vectors

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Implementing Quality by Design (QbD) principles early during product development is critical to ensure commercial success for therapeutics. QbD requires understanding the quality target product profile (QTPP) ahead of product development to determine the quality attributes of the final product. Critical quality attributes (CQAs) of potency, purity, identity, and safety are then determined through a risk assessment of these attributes. Beyond risk management, robust analytical methods are necessary to measure these attributes and present a clear control strategy to regulatory agencies. The ACTX-101 process was developed as a scalable suspension system with agnostic downstream operations platformed for AAV9 serotype vectors. Utilizing the principles of QbD, product-specific impurities were identified as key critical quality attributes. These product-specific impurities include: encapsidated plasmid and host cell DNA, empty and partially filled capsids, aggregated/deamidaded/glyclosylated forms, and replication-competent AAV. We will present a case study specific to these CQAs and their impact on potency and safety.

1052 DRIMER: Optimized Programmable RNA Scaffolds for Multivariate Multiple and Different Effector Recruitment Using CRISPR/dCas9

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Rational engineering of RNA has enabled diverse applications ranging from COVID vaccines to improved guide RNA (gRNAs) for CRISPR/Cas systems *in vivo* and *in vitro*. For instance, nearly all CRISPR/Cas9-based genome editing applications rely upon gRNAs that are synthetic fusions between the CRISPR and trans-activating CRISPR RNAs (crRNAs and tracrRNAs, respectively). Further, chemical modifications to gRNAs are often required for genome editing in cell and gene therapy contexts as well as CRISPR activation (CRISPRa) strategies. gRNAs are

frequently modified to incorporate stem-loops that can recruit RNA binding proteins fused to different transcriptional effectors. However, these stem-loop platforms currently offer limited spatiotemporal control and can sometimes even interfere with gRNA efficacy. Here we developed and optimized a designer RNA intermediated effector recruitment platform, called DRIMER that permits robust stochiometric and spatiotemporal control over effector recruitment in CRISPR/Cas systems. DRIMER can recruit up to four different effector domains to human loci. Importantly, these effectors can be functionally distinct and recruited in user-defined combinations. For instance, using the DRIMER system, we demonstrated powerful synergistic effects following the recruitment of multiple synthetic transcription factors and epigenetic editors with respect to transcription activation from human promoters. Overall, the DRIMER platform is a highly programmable system that allows stoichiometric and combinatorial effector recruitment for precise modulation of human gene expression, which is an attractive capability for a wide range of cell and gene therapy applications.

1053 Characterizing the Phenotypic Abnormalities of a Novel Mouse Model of Snyder-Robinson Syndrome: For Therapeutic Development

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Background: The polyamines putrescine, spermidine, and spermine are essential molecules for cellular growth processes such as gene expression, signal transduction, and ion channel regulation. Accumulation of spermidine due to mutations in the *SMS* gene, encoding spermine synthase, SMS, causes Snyder-Robinson syndrome (SRS). This rare X-linked recessive disorder, with no available treatment, manifests with a spectrum of symptoms including intellectual disability, developmental delay, seizure, thin habitus, and low muscle tone. Therapeutic development for SRS requires an understanding of the disease pathophysiology in a suitable disease-specific animal model that recapitulates the abnormalities seen in patients. A mouse model carrying a missense mutation in the *Sms* gene resulting in a glycine-to-serine substitution at position 56 (G56S) of SMS protein, was recently generated. This mouse was modeled after patients with a severe form of SRS. Our aim is to characterize the disease manifestation in the G56S mouse as a model for SRS and to study the molecular mechanisms underlying SRS pathogenesis. Similarly, we aim to develop a gene therapy approach for SRS using this mouse. We hypothesize that *Sms* mutation in the G56S mouse leads to abnormal phenotypic signatures that mimic the disease presentation in patients. **Methods and Results**: We performed molecular, behavioral, and neuroanatomic studies to evaluate neurological functions and to understand the pathophysiology of SRS in the G56S mice. We observed a complete loss of SMS protein and elevated spermidine/spermine ratio in the mutant mouse tissues. In addition, the G56S mice had increased anxiety, signs of impaired learning, and decreased explorative behavior, as assessed using fear conditioning, Morris water maze, and open field tests respectively. Furthermore, the mice failed to gain weight over time and exhibited abnormalities in brain structure and bone density on T2-weighted MRI and micro-CT scans respectively. Transcriptomic analysis of the brain cortex of the mutant mice indicates downregulation of genes involved in mitochondrial oxidative phosphorylation and ribosome protein synthesis. Similarly, isolated fibroblasts from the G56S mouse showed impaired mitochondria bioenergetic, suggesting a possible role of impaired mitochondria respiration in disease pathogenesis. **Conclusion**: Our data showed the G56S mouse recapitulates many of the SRS phenotypic abnormalities and revealed key cellular and molecular processes that are altered in the mice that could possibly contribute to the disease pathogenesis. Current experiments focus on the development and evaluation of a gene therapy approach for SRS via viral-mediated expression of the WT *Sms* gene. We anticipate that the successful completion of this study will open the door to a gene therapy approach for SRS and other polyamine-related genetic diseases.

1054 Generation of Genetically Engineered T Cells Expressing Affinity-Enhanced TCR Anti-NY-ESO-1:HLA-A*02 with Potent and Target-Specific Antitumor Activity for the Treatment of Solid Tumors

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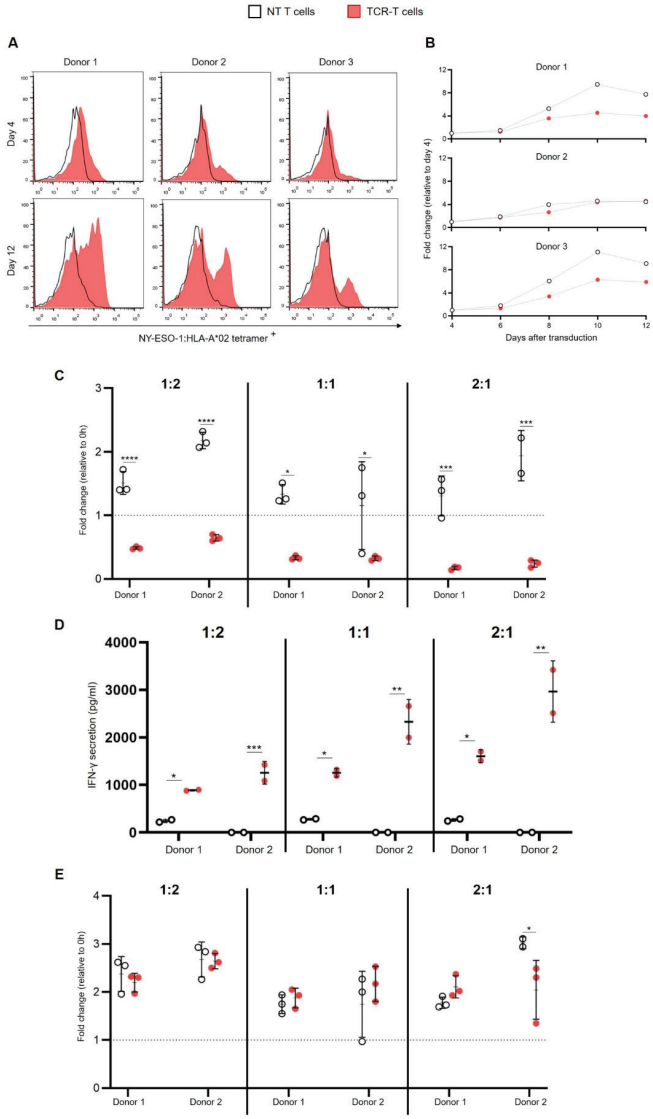
Impressive clinical responses have been achieved in CAR-T cell therapy for hematologic neoplasms. These cells efficiently eliminate tumor cells through the restricted recognition of surface antigens. However, most tumor-associated antigens are intracellular, thereby restricting the application of classical CARs against solid tumors, which represent the vast majority of the neoplasms. To overcome this limitation, T cells engineered to express artificial T cell receptors (TCRs), or TCR-T cells, are able to recognize intracellular proteins presented by the major histocompatibility complex (MHC, or HLA in humans), expanding the options of therapeutic targets. In this context, the NY-ESO-1 is a target that stands out for its restricted expression in healthy tissues (placental and immune-privileged organs) and strong and homogeneous expression in several types of tumors. Thus, the main goal of this study was the generation of TCR-T cells expressing an affinity-enhanced TCR against the NY-ESO-1:HLA-A*02 complex aiming at developing an innovative advanced cell therapy product in Brazil, to be made available through the public health system. To that end, human T cells (3 donors) were transduced with a lentiviral vector for expression of a recently developed new affinity-enhanced TCR targeting the NY-ESO-1:HLA-A*02 complex. The transduction efficiency accessed by labeling with NY-ESO-1:HLA-A*02 specific tetramers was on average of ~17% (± 5.1%) on day 4 and increased ~1.5 times on day 12, reaching an

average of ~25% (± 8.4%) (figure 1A), indicating an enrichment of the T cell population expressing the artificial TCR. In vitro growth kinetics of transduced T cells were similar to that of non-transduced T cells (NT T cells) (figure 1B). Next, cytotoxicity and selectivity of TCR-T cells (2 donors) were evaluated in a co-culture assay with SW982^{luc} cells (human synovial sarcoma cells, NY-ESO-1^{+}, HLA-A*02^{+}) or HCT116^{luc} cells (NY-ESO-1^{-}, HLA-A*02^{+}). TCR-T cells were able to eliminate on average 43%, 68% and 80% of the SW982^{luc} cells after 48h of co-culture in 1:2, 1:1 and 2:1 effector-to-target ratios, respectively (figure 1C). SW982^{luc} tumor cell lysis was accompanied by increased secretion of IFN-γ when compared to NT T cells (figure 1D). Additionally, TCR-T cells had no cytotoxic capacity against HCT116^{luc} cells, demonstrating their target-specific activity (figure 1E). Taken together, these results are promising in view of the development of a new advanced cell therapy product for patients with NY-ESO-1-positive tumors. **Figure 1A**. T cells transduction efficiency. **B**. Cellular proliferation rate. **C.** Co-culture with SW982^{luc} cells (bioluminescence). **D.** IFN-γ secretion after co-culture with SW982^{luc} cells. **E.** Co-culture with HCT116^{luc} cells (bioluminescence). ANOVA, multiple comparisons; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).



1055 Utilizing Surface-Functionalized Nanoneedles for *Ex Vivo* Physical Cargo Delivery into Cells

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The development of cell and gene therapies can be impacted by deleterious and unintended effects on cells and patients from the current suite of available delivery methods. Fragile cell types are often destroyed during electroporation and fine dosage control is restricted. Strict constraints on cargo size, manufacturing capacity issues and the potential for unexpected deleterious effects within patients make viral vector-based methods also challenging. Microinjecting cells one-by-one is tedious, and the process can often kill cells. Lipid and polymer-based methods have low reproducibility, cytotoxicity issues, low efficiency in many different cell types and cannot support complex or multiplexed cargos. This lack of safe and effective delivery prevents utilizing engineered cells from widespread therapeutic use, despite showing incredible promise in curing previously untreatable diseases. Here we describe the next generation of molecular payload delivery, a novel *ex vivo* method employing an array of engineered silicon nanoneedles on a chip that enables direct intracellular access in a precise, efficient, and automated manner. We pair our nanoneedle technology with an aptamer-based surface chemistry that controls discharge of the desired biomolecule directly into the cell. Our cell engineering platform can deliver precisely to many cells at once and has eliminated many of the issues with the preceding conventional delivery methods. Our nanoneedle delivery approach has displayed positive results with an assortment of cell and cargo types, allowing us the ability to deliver nucleic acids, proteins, and RNP complexes into a range of immortal, stem, and primary cell types with higher viability and efficiency than current industry standard methods. This platform has remarkably exhibited single cell precision at scale, and the ability to multiplex with dosage control. We believe our nanoneedle delivery system will unlock previously unobtainable engineered cell therapies with its uniquely promising capabilities.

1056 AAV Packaging in HEK293 Cells Prefers Unit-Length Vector Genomes

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Recombinant adeno-associated virus (rAAV) is a successful gene therapy vector providing FDA-approved therapies. Generally, AAV has a normal packaging capacity of ~4.7kb, but around 5.3kb genome can also be efficiently packaged. In sf9 cells, full-length and oversize genome has been reported regardless of vector unit length, which leads to the increase sf9-produced vector heterogeneity. Here we systemically investigated if non-unit length packaging AAV genomes occurs in HEK 293 cells. First, four vector plasmids containing rAAV genome with a size of 848bp (AAV8-TTR-coFVIII-848), 1320bp (AAV8-TTR-coFVIII-1320), 2981bp (AAV8-TTR-coFVIII-2981) or 4707bp (AAV8-TTR-coFVIII-4707) were used for vector production in the

presence of a helper plasmid (pFΔ6, encoding adenovirus E4, E2A and VA) and a Rep-Cap plasmid (pH28) into suspension HEK293 cells to make rAAV8 vectors. The vectors were purified by iodixanol gradient ultracentrifugation. The resulting vector particles were analyzed using charge detection mass spectrometry (CD-MS). Interestingly, peak fractions of both AAV8-TTR-coFVIII-848 and AAV8-TTR-coFVIII-1320 vector preparations are either empty particles or particles with varying amounts of unit-length genomes. There was a positive correlation between vector mass weight and its genome size (r=0.999); In contrast, vectors from AAV8-TTR-coFVIII-2981 and AAV8-TTR-coFVIII-4707 showed only empty particles and the full particles containing a single unit length genome. There was a broader distribution of partial particles in AAV8-TTR-coFVIII-4707 vector preparations, and no oversized packaging of unit length genome was observed. Alkaline agarose gel electrophoresis data indicates the that the AAV8-TTR-coFVIII-848 vector packaged DNA distributions at sizes 848, 1696, 2544, 3392, 4240 and 5088bp. In addition, the AAV8-TTR-coFVIII-1320 vector the packaged DNA distributions at sizes 1320, 2640, 3960 and 5280bp indicating that this kind of shorter AAV genome was packaged in a unit-length DNA ligation way. In contrast, the AAV8-TTR-coFVIII-2981 and AAV8-TTR-coFVIII-4707 vectors showed a single band at the corresponding unit length vector genome. These results demonstrated that AAV packaging in HEK293 cells has a preference for encapsidating a genome close to its maximal capacity by packaging multiple unit-length genomes when unit-length genome is very small, in contrast, non-unit length genome packaging was inhibited. Taken together, AAV packaging behavior in HEK 293 cells is different from what has been reported in sf9 cells. There might exist different packaging mechanisms between the two types of cells.

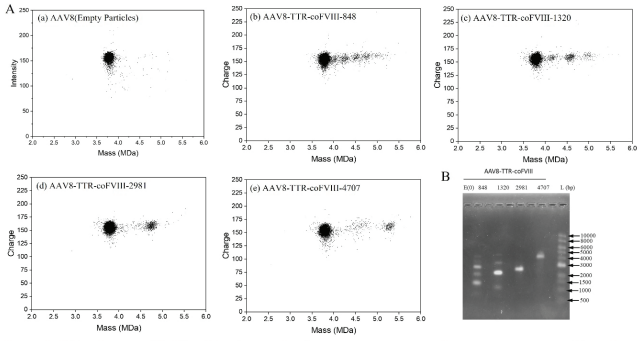

Figure 1. AAV packaging in HEK293 cells prefers unit-length vector genomes. A(a-e), Charge versus mass scatterplots for the vector genomes. B, Alkaline gel measurements of ssDNA from the vectors

1057 Gene Activation Mediated by Zinc Finger Transcriptional Regulators (ZF-TRs) as a Therapeutic Approach for CNS Disorders

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Neurodevelopmental disorders are impairments of the development of the brain and/or central nervous system (CNS) that affect emotion, learning ability, self-control, and memory. The development of the nervous system is tightly regulated and is influenced by both genetic and environmental factors. Some neurodevelopmental disorders are considered multifactorial syndromes which have many causes that converge to a more specific neurodevelopmental manifestation in forms of haploinsufficiency. At Sangamo Therapeutics, we are utilizing our proprietary zinc finger transcriptional regulator platform (ZF-TR) to upregulate gene expression by tethering zinc fingers to a trans-activation domain. Here we demonstrate target upregulation at mRNA and subsequently protein levels *in vitro* (mouse cortical neurons and CDI human iPSC-derived neurons) and *in vivo* (wildtype mouse). We successfully demonstrated *in vitro* to *in vivo* translatability by injecting our zinc finger activators to wildtype mice. Thus, Sangamo's zinc finger activator platform could be utilized to potentially alleviate reduced levels of functional proteins in neurodevelopmental disorders caused by haploinsufficiency.

1058 Temsirolimus and IL-7 Treatment Synergistically Increase Primary Resting CD8+ T Cell Transduction with CD8-Targeted Fusosomes and Enhance CD19CAR Expression

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Introduction: Resting human T cells are difficult to transduce with lentiviral vectors (LVV) primarily due to blocks imposed by cellular restriction factors during viral replication. These blocks collectively limit the potency of LVV-based T cell gene therapies. While restriction factors have been extensively studied in CD4+ T cells infected with HIV-1, the impact of these proteins in CD8+ cells is not as well-described. We investigated the role of known restriction factors in limiting targeted lentiviral vector (fusosome-LVV) mediated transduction in primary CD8+ T cells and have found that IFITM1 and SAMHD1 impose potent blocks in this cell type. Mechanistically, IFITM1 impedes LVV fusion at the cell membrane while SAMHD1 interferes with reverse transcription by cleaving cellular dNTPs and reducing available nucleotide pools. We hypothesized pharmaceutical intervention(s) that have been shown to inactivate these restriction factors may increase potency, potentially reducing the minimal efficacious dose of fusosome-LVV. Previous data have shown that IFITM1 can be downregulated by pre-treating resting T cells with rapamycin, while IL-7 pre-treatment can lead to enhanced levels of the inactive form of SAMHD1 (pSAMHD1). Having identified both factors as being potent restriction factors in CD8+ T cells through restriction factor knockout experiments, we developed an optimized combination treatment including IL-7 and temsirolimus (TEM), a rapamycin analog (rapalog), that dramatically improves gene delivery efficiency and downstream efficacy of our CD8-targeted fusosome-LVVs *in vitro*, thus demonstrating that overcoming restriction factors in cell and gene therapy may enhance the efficacy of these viral vectors.
Methods/Results: Resting pan-T cells from multiple donors were pre-treated for 3 days with IL-7 or IL-2 as a control. Cells were then incubated with TEM for 2 hours before a 2-hour transduction with a

range of CD8-targeting fusosome-LVV delivering GFP or CD19CAR transgenes. Cells were washed and left in IL-2/IL-7 containing media for 1 day before CD3/CD28 activation. Six days later, cells were analyzed by FACS for GFP or CAR expression. CAR T cell function and ability to kill Nalm6 tumor cells was also assessed. The combination of TEM and IL-7 pre-treatment led to the greatest dose-dependent increase in transduction efficiencies in resting T cells in all donors as measured by FACS with higher CD19CAR or GFP expression observed with IL-7+TEM compared to IL-2+TEM treatment. Furthermore, IL-7 and TEM synergistically improved transduction efficiencies and GFP expression up to 11X compared to cells incubated with either component alone. Varying the pre-treatment IL-7 dose did not lead to dose-dependent differences in transduction efficiencies by FACS. Western blot analysis of IFITM1 and pSAMHD1 protein levels highlight a specific effect of combination treatment on relevant restrictions factors; a pronounced loss of IFITM1 protein expression after 2 hours of TEM treatment and an increase in pSAMHD1 levels after 3 days of IL-7 pre-treatment was observed across multiple donors. **Conclusions:** Our findings identify key restriction factors that limit transduction by our CD8-targeted fusosome-LVVs and show that potency is significantly increased in resting T cells following IL-7 and TEM treatment in a transgene-independent manner. This increase in potency is consistent with the inactivation or loss of two critical restriction factors, SAMDH1 and IFITM1, as demonstrated by western blot analysis. Collectively, these results suggest a strategy toward a combination *in vivo* vector-based therapy that is supplemented with pharmaceutical agents to improve vector potency.

1059 Multiplex Knockdown of ANGPTL3 and CYPOR as a Novel Treatment for Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is a genetic disorder characterized by elevated low-density lipoprotein cholesterol (LDL-C) resulting in premature atherosclerotic cardiovascular disease. The knockdown of ANGPTL3 using CRISPR-Cas9 gene editing has been explored as a novel approach for treating FH. Ex vivo gene editing is potentially safer than in vivo approaches to treat genetic diseases affecting the liver. Still, low engraftment by gene-edited hepatocytes after transplantation poses a challenge for this ex vivo strategy. By knocking down NADPH-cytochrome P450 oxidoreductase (CYPOR) followed by treatment with APAP, a selective advantage is given to gene-edited cells after transplantation. A novel gene editing approach for treating FH includes isolating hepatocytes from the patient's resected liver, editing hepatocytes ex vivo with Cas9 and Cas12a systems to knock down ANGPTL3 and CYPOR, and transplanting the gene-edited cells back into the patient. This procedure would be followed by transient APAP administration to select edited cells in vivo. In contrast to Cas9, multiplex gene editing using the Cas9/Cas12a system will reduce the potential for chromosomal rearrangement. Here, we optimize multiplex gene editing for Angptl3 and CYPOR using both dual Cas9 and Cas9/Cas12a systems in Hepa1-6 cells and primary mouse hepatocytes. In preliminary in vivo experiments, we delivered *Cypor*-targeting plasmid via hydrodynamic injection and treated the mice biweekly with APAP to produce an ALT elevation >1000 IU/L. After repeated injections, the ALT decreased to normal levels in the experimental group. This decrease indicated the successful selection and expansion of APAP-resistant *Cypor*-deficient hepatocytes. We achieved an average of 45% *Cypor*-knockdown in the livers of APAP-selected C57bl6 mice. Next, we delivered *Cypor*-targeting Cas9 RNPs to hepatocytes ex vivo via electroporation and transplanted the edited hepatocytes into C57bl6 mice. When the APAP selection process was repeated, we observed an ALT peak followed by a decrease. At the end of the experiment, we observed an average engraftment of 15% in the experimental mice compared to 2% in the control and no indications of liver failure. In Hepa 1-6 cells electroporated with *Angptl3*- and *Cypor* - aiming Cas9 RNPs, we observed 83% and 92% indels for *Angptl3* and *Cypor,* respectively. In Hepa1-6 cells electroporated with *Cypor*-aiming Cas9 RNPs combined with *Angptl3*-aiming Cas12a RNPs, the optimized condition resulted in 78% and 90% indels for *Angptl3* and *Cypor*, respectively. **Conclusion** Our findings suggest that *Cypor* knockout followed by APAP treatment is a promising method to select for the gene-edited hepatocytes. Moreover, this selection method could be successfully combined with an electroporation-mediated ex vivo gene editing strategy. This study also provided preliminary data showing multiplex delivery of Angtpl3- and Cypor-aiming sgRNA with Cas proteins offers high levels of gene editing in a cell line using both a dual Cas9 and Cas9/Cas12a system. In future studies, we will demonstrate proof-of-principle multiplex editing to knock down *Cypor* and *Angplt3* combined with APAP selection for reversing atherosclerosis in Ldlr-/- mice and evaluate the safety of dual Cas9 and Cas9/Cas12a multiplex gene editing.

1060 Improved Differentiation Efficiency and Proliferation Ability of NK Cells from Cord Blood HSCs by Hyperosmosis

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Natural killer (NK) cells are cytotoxic innate lymphoid cells with great potential for immunotherapy. Engineered NK cells to express a CAR are an alternative immunotherapeutic strategy to CAR-T cells for cancer treatment. NK cells from PB cells can only be expanded hundreds or thousands of times, and this drawback can be overcome by stem cell-derived NK cells. iPSC-derived NK cells (iPSC-NK) are a potential source for CAR-NK cells, but it takes several months for gene modification, clone selection, and directed differentiation. Here we show that cord blood hematopoietic stem and progenitor cells (HSPCs) can be expanded and differentiated into a large number of

HSC-NK cells in less than four weeks. We developed a protocol that includes differentiation of HSPCs on OP9 feeder cells transduced with human DLL1 and DLL4 with high osmotic pressure. Hyperosmosis can be achieved by adding NaCl or PBS to the medium. **Results:** Compared with normal osmotic pressure (300 mM), the high osmotic pressure of 330 mM significantly increased the efficiency of NK cell differentiation (**Figure 1**). Further expansion on the K562-mb21 feeder led to a greater purity of $CD3^{-}CD56^{+}$ NK cells from HSC-NK than that of PB-NK cells. In addition, high osmotic pressure-primed HSC-NK cells showed enhanced proliferative activity with 1.8×10^{6}-fold total expansion and an 11-fold increase in total yield compared to the control (**Figure 2**). Of interest, we found that activating receptors, such as CD69, NKp30, and NKp44, were significantly increased in HSC-NK-300 mM or HSC-NK-330 mM cells compared with PB-NK cells. RNA-seq analysis showed that HSC-NK cells expanded under normal or high osmotic pressure were virtually indistinguishable. Furthermore, the antitumor activities of HSC-NK cells against HepG2 hepatocellular carcinoma cells, MOLM-13 AML cells, and SKOV-3 ovarian cancer cells were comparable to or moderately greater than the PB-NK cell control. Together, hyperosmosis-induced HSC-NK cells are similar to expanded PB-NK cells in phenotype, transcriptome, and killing activities against tumor cell lines. **Conclusions:** Our study supports the possibility of the high-yield generation of NK cells for clinical therapy. Together with lentiviral or CRISPR-mediated CAR integration, our approach will generate sufficient CAR-NK cells from one cord blood for hundreds, even thousands of patients in one month. The finding of hyperosmosis-facilitated NK cell differentiation should find applications in the iPSC-NK generation.

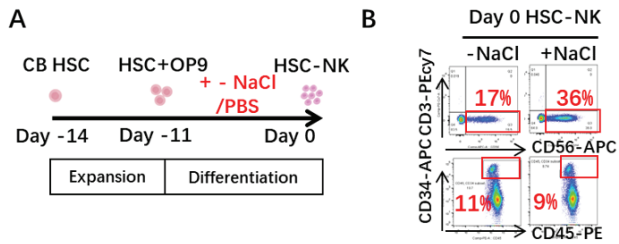

Figure 1. High osmotic pressure promotes NK cell differentiation.

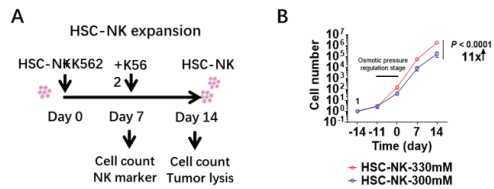

Figure 2. High osmotic pressure-primed HSC-NK cells show enhanced proliferation activity.

1061 Unconstrained Mitochondrial DNA Base Editing Enables Precise Disease Modeling

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The human mitochondrial genome is a 16,569 bp long, multicopy, circular double-stranded DNA (dsDNA) molecule that encodes 37 genes essential for cellular energy metabolism. Pathogenic mitochondrial DNA (mtDNA) variants are prevalent in ~1 in 8,000 people and are causal in incurable metabolic disorders. Current mtDNA editing strategies rely on all-protein systems, such as transcription activator-like effector (TALE)-based technologies. In general, these technologies are composed of a TALE, which acts as a programmable DNA-binding domain, fused to an effector domain. In particular, dsDNA deaminase A (DddA)-derived cytosine base editors (DdCBEs) consist of TALE-$DddA_{tox}$ fusions. Given its preference for dsDNA, $DddA_{tox}$, the deaminase domain of DddA, is split into two inactive halves to avoid toxicity. Thus, a DdCBE monomer incorporates either the N- or C-terminus of split $DddA_{tox}$ downstream of a TALE. In the context of DdCBE pairs, binding of their respective DNA-binding domains to adjacent target sequences enables the reassembly of functional $DddA_{tox}$, followed by targeted cytidine deamination. Then, uracil glycosylase inhibitors positioned downstream of the split $DddA_{tox}$ halves impede the excision of the resulting uracil residues. Subsequently, U•G intermediates are resolved into T•A base pairs during mtDNA replication, which occurs even in post-mitotic cells. This process results in programmed C•G-to-T•A conversions in mtDNA. Despite their flexibility and robustness, the versatility of canonical DdCBEs is limited by the requirement of a thymine immediately upstream of their respective TALE target sequences. This double 5'T constraint (one per DdCBE monomer) can be difficult or impractical in certain contexts. Based on our previous work on the FusX TALE Base Editor, a platform for the rapid design and assembly of mitochondrial base editors, we generated improved DdCBEs that bypass the 5'T requirement. First, seeking to characterize the activity and specificity profiles of improved DdCBEs relative to their canonical counterparts, we generated DdCBE pairs that target sequences preceded by 5'T or 5'V (V = A, C, G) in both canonical and improved formats. Hence, for a single locus, we tested four DdCBE pairs: a canonical or improved 5'T-compliant pair, and a canonical or improved 5'T-uncompliant pair. We chose four mtDNA loci to test this strategy: *MT-ATP6*, *MT-CO1*, *MT-ND2*, and *MT-ND4*. Thus far, we have not observed significant differences between the activities of 5'T-compliant canonical vs. improved DdCBEs. Additionally, utilizing improved DdCBEs, we installed the pathogenic m.3242G>A variant with no bystander editing at the *MT-TL1* locus in HEK293T cells, demonstrating their potential for precise mitochondrial disease modeling. In summary, we are evaluating the efficiency and specificity of improved DdCBEs that bypass the 5'T requirement, which will support unconstrained mtDNA base editing as a strategy for disease modeling and gene therapy applications.

1062 Estimating Probability of Success of Gene and Cell Therapy Development Programs

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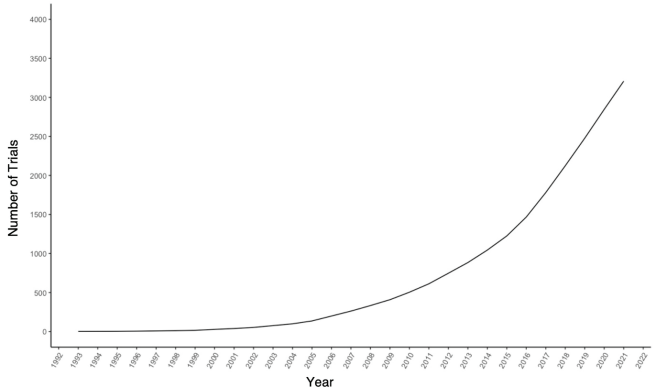
Introduction: Gene and cell therapies (GCTs) are a rapidly growing class of pharmaceuticals that have emerged over the past two decades and have shown to hold particular promise for rare diseases and other conditions with unmet medical needs. Despite their therapeutic potential, the development of GCTs faces a number of unique challenges related to complex manufacturing and regulations, as well as exceptionally high costs. An increased understanding of development pathways and clinical trial trajectories could support increased efficiencies and improved forecasting of research and development costs. Here, we analyze the clinical development landscape of CGTs to provide the overall success rates and unravel favorable features that increase the probability of success. **Methods:** We used AdisInsight, a pharmaceutical intelligence database, to identify all clinical trials of GCT products entering commercial clinical development worldwide between January 1990 and July 2022. GCT products were identified through the hierarchal search function of the database. We defined a development path as testing a product in a specific indication. Therefore, a single trial can constitute multiple development paths if multiple indications were tested. To estimate a product's probability of success (POS) in reaching marketing approval in any jurisdiction, we used previously developed methods and modeled a product's development pathway using imputation to estimate missing trial phases (Wong C. H., et al., (2019), Biostatistics). A development program was considered terminated if the time elapsed after the conclusion of the clinical trial was 360 days for phase I, 540 days for phase II, and 900 days for phase III and no trial in the next phase has started. To estimate the change in POS over time, we performed a competing risk analysis, using the time from initiation of clinical development to approval or termination (Putter, H., et al., (2007), Statist. Med.) **Results:** There were 3369 clinical trials that represented 4386 development paths for 1311 GCT products starting commercial development between 1993 and 2022 (Fig.1). 50 (3.8%) GCT products were approved worldwide for 64 indications. Most trials were phase I or phase I/II, with phase III constituting less than 10% of the conducted trials. The majority of trials studied oncology indications, followed by cardiology and endocrinology. On average each product was tested in 3 indications, while oncology products were tested on average in 4 indications. The overall POS for GCT products from phase I to approval was less than 5%. We observed that the use of regulatory incentives such as orphan designation and fast-track increased the POS. Considering POS as a function of time, POS peaked at 15 years after initiation of clinical development with most program terminations occurring in the first 5 years. **Conclusion:** There has been a rapid increase in clinical trials evaluating potential GCT therapies over the past decade. Several trial features, including the use of regulatory incentives, appear to be associated with higher POS and may represent important considerations in designing GCT development programs and guiding early decisions.

Figure 1: Cumulative increase in clinical trials for gene and cell therapies, 1993-2022



1063 AI-Driven Design of RNA-Based Gene Control Systems

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Gene therapies that suffer from clinical holds and failures often cite off-target effects, where the therapy is active in unintended tissues, as a major reason. The FDA has recently highlighted issues of adverse off-target effects in tissues including the liver, kidney and motor neurons. As such, the cell selectivity of gene therapies is becoming increasingly important. Although technologies such as synthetic promoters and tropic vectors have addressed some of the major safety concerns, effective control systems remain difficult to build, with development involving extended trial-and-error processes with no guarantee that an effective control system will be produced. We are developing RNA-based gene control systems to control the activity of cell and gene therapies in a defined manner. Our approach will enable the control of therapeutic activity in response to various conditions (e.g. cell/tissue type, disease, cell differentiation), including FDA approved small molecule drugs, which is typically not possible with current generation cell and gene therapies. Our control systems are RNA sequences that form part of the cell/gene therapy cassette and, in contrast to other control systems, require no additional molecular machinery, avoiding associated complications such as increased immunogenicity, unpredictability of therapeutic efficacy/safety, and poor translation of animal data into humans. Notably, existing control systems only respond to a handful of inputs with limited commercial utility, and building systems that respond to new commercially relevant inputs is very difficult. Current methods are highly inefficient, requiring the testing of 100,000s of candidate sensors while only covering <1 billionth of the design space. Our AI-led design process can explore the design space in a more intelligent and controlled manner, reducing the required amount of testing >10-fold and massively increasing success rates. Our high-throughput experimental platform provides incredibly information-dense data in a highly parallelised manner while requiring minimal time and resources to perform, allowing us to test control system candidates at scale and rapidly identify promising candidates. We are currently using these methods to develop disease and tissue specific control systems. The key benefit of this approach is that the resulting control systems are therapeutic agnostic and can be incorporated into any type of gene therapy, including cell and RNA

therapies, and are compatible with all delivery mechanisms. This approach will improve safety and efficacy by reducing the likelihood of adverse effects and allowing active control of dosage, thereby enabling refined clinical management and monitoring.

1064 Healthcare Utilization, Burden of Illness, and Mortality in Adults with Adrenomyeloneuropathy: The Case for Genetic Therapies

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Background: Adrenomyeloneuropathy (AMN) is an X-linked neurodegenerative disease caused by mutations in *ABCD1* resulting in progressive myeloneuropathy that causes spastic paraparesis, sensory ataxia, loss of mobility, incontinence, and sexual dysfunction. Disease onset is earlier in men compared to women who are heterozygous for the disease. The burden of illness and impact on healthcare resource utilization (HRU) have yet to be described. We quantified HRU, clinical characteristics, and mortality in adults with AMN in the US. **Design/Methods**: HRU was assessed using commercial insurance claims from IQVIA's PharMetrics Plus database (1/01/2006-6/30/2021). AMN patients were 18-64y with ≥1 inpatient or ≥2 outpatient claims containing an AMN diagnosis (ICD-10-CM: E71.52x; ICD-9-CM 277.86), no evidence of childhood cerebral adrenoleukodystrophy, other peroxisomal disorders, or pregnancy/labor or delivery. AMN patients were propensity score matched to non-AMN individuals on demographic characteristics. Patients were followed for as long as they were observable. Separately, mortality rates and age at death were assessed in the Medicare Limited Dataset (1/01/2017-03/25/2022) for AMN patients and compared with all beneficiaries.**Results**: We identified 806 commercially insured AMN patients with a mean age of 35.0 ±12.44y, followed for an average 22.7±23.55 months. AMN patients had more inpatient admissions (0.30 vs. 0.05); outpatient clinic (10.64 vs. 5.54), outpatient hospital (4.65 vs. 1.19), and home healthcare visits (2.88 vs. 0.21); more durable medical equipment claims (0.52 vs. 0.10); and more prescription medications (18.36 vs 7.84 pharmacy fills) per patient per year than non-AMN controls. Hospital length-of-stay (8.23 vs. 4.89 days) was also longer in AMN patients. All reported HRU comparisons were statistically significant, $p<.05$. Comorbidities were more common in AMN compared to controls, including peripheral vascular disease (3.1% vs. 0.6%), chronic pulmonary disease (6.1% vs. 3.2%), and liver disease (4.1% vs. 0.8%), all $p<.05$. Comparing AMN with controls throughout - mortality rates among younger (ages 18-64y) AMN Medicare enrollees were 5.3x higher among males (39.3% vs. 7.4%) and 3.2x higher among females (15.7% vs. 4.9%), both $p<.001$. Age at death was significantly younger for male AMN patients 18-64y (47.0±11.3 vs. 56.5±7.8, $p<0.001$). Among AMN patients ages ≥65y, mortality rates were 2.2x higher for males (48.6% vs. 22.4%) and 1.5x higher for females (31.2% vs. 20.3%), both $p<.001$. **Conclusions**: AMN imposes a substantial and previously under-recognized health burden for adults with men affected earlier and more severely than women, including greater medical comorbidities, higher healthcare utilization, higher mortality rates, and, in some subgroups, younger age at death. The high unmet need and genetic disease burden calls for disease-modifying interventions and points to the value in developing targeted genetic therapies.

Patient Demographic and Clinical Characteristics

Characteristics	Cases (AMN) n = 806	Controls (Non-AMN) n= 2726
Demographics, n (%)		
Follow-up months, mean ± SD	22.72 ± 23.55	25.32 ± 23.92
Age, mean ± SD	35.04 ± 12.44	34.92 ± 12.18
Age - 18-35	493 (61.2%)	1654 (60.7%)
Age - 36-51	197 (24.4%)	700 (25.7%)
Age - 52-64	116 (14.4%)	372 (13.6%)
Female	503 (62.4%)	1689 (62.0%)
Comorbid Conditions, n (%)		
Charlson Comorbidity index, mean ± SD	0.46 ± 1.11	0.17 ± 0.65
Non-Diabetic Neuropathy	73 (9.1%)	19 (0.7%)
Chronic Pulmonary Disease	49 (6.1%)	87 (3.2%)
Hemiplegia/Paraplegia	49 (6.1%)	<5
Liver Disease	33 (4.1%)	23 (0.8%)
Diabetes	30 (3.7%)	106 (3.9%)
Peripheral Vascular Disease	25 (3.1%)	17 (0.6%)
Cerebrovascular Disease	25 (3.1%)	11 (0.4%)
Cancer	23 (2.9%)	31 (1.1%)
Renal Disease	15 (1.9%)	12 (0.4%)
Congestive Heart Failure	12 (1.5%)	8 (0.3%)
Rheumatic Disease	12 (1.5%)	23 (0.8%)
Multiple Sclerosis	10 (1.2%)	5 (0.2%)
Peptic Ulcer Disease	8 (1.0%)	5 (0.2%)
Diabetic Neuropathy	8 (1.0%)	11 (0.4%)
Dementia	6 (0.7%)	<5
Myocardial Infarction	5 (0.6%)	6 (0.2%)
HIV/AIDs	<5	<5

Measures containing less than 5 patients are masked to protect patient confidentiality

1065 Chemically Modified Single-Stranded DNA Donors Enable Efficient mRNA Gene Editing-Mediated Knock-In in Human iPS Cells

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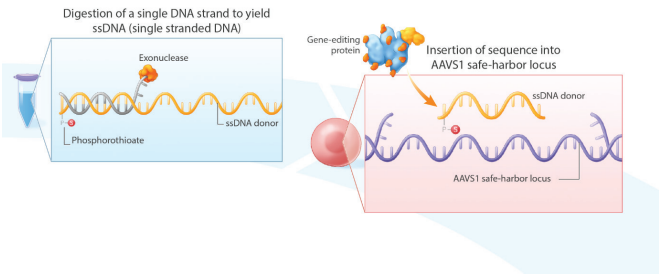
Genome-editing technology provides a means of modifying genes in living cells, and is being explored for the development of therapies to treat cancer and a variety of genetic disorders. Gene-editing proteins can be used to create single- and double-strand breaks at specific genomic sites for knocking out a gene or, when combined with an exogenous DNA donor, knock-in of a defined sequence. Compared with double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) exhibits lower toxicity and is less prone to random genomic integration, making it an attractive form of DNA donor for gene knock-in. However, current methods of synthesizing ssDNA, including enzymatic digestion, asymmetric PCR, and chemical synthesis, suffer from low yields, contamination with residual dsDNA, length limitations, and high cost. Here, we present an enzymatic approach for producing long (>3kb) and concentrated (>1μg/μL) ssDNA suitable for generation of knock-in lines of human induced pluripotent stem (hiPS) cells. We PCR-amplified dsDNA from plasmid templates using 5'-modified primers incorporating a 5' phosphate on the strand intended for digestion and a single 5' phosphorothioate linkage on the protein-

encoding strand to protect against digestion. The resulting PCR products were treated with lambda exonuclease, which preferentially digests strands with a 5' phosphate group, to yield a single-stranded product. To minimize degradation of the ssDNA, we included in the reaction a short, double-stranded oligonucleotide (dsDecoy), for which lambda exonuclease has higher affinity than ssDNA. Reactions were further treated with the less processive T7 exonuclease to eliminate residual dsDNA not digested by lambda exonuclease, yielding concentrated (>1µg/µL) ssDNA. Gel electrophoresis analysis of five products ranging from 1.4kb to 3.3kb revealed a single, sharp band in the region of interest. Four of the ssDNA products contained less than 0.3% residual dsDNA by mass, as determined by gel electrophoresis using a double-stranded standard of known concentration. The fifth, 3.3kb ssDNA product contained 1.1% dsDNA by mass. To test their utility as knock-in donors, ssDNA products were co-electroporated into hiPS cells with mRNA encoding UltraSlice gene-editing proteins targeting the AAVS1 safe harbor locus. Insertion efficiencies were 67.8% for a 1.2kb donor, 8.6% for a 2kb donor encoding a ROR1 CAR, and 2.7% for a 2.8kb donor encoding green fluorescent protein. In summary, we demonstrate a method for high-yield synthesis of ssDNA suitable for cellular applications, including mRNA gene editing-mediated knock-in in iPS cells. Overall, this platform may prove useful in the development of gene-editing therapeutics.



1066 Performance and Modularity of Horizon's Pin-point™ Base Editing System Characterized by Arrayed and Pooled Screening Platforms

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Base editing is a CRISPR-Cas-based technology that allows the introduction of point mutations in the DNA without relying on double strand break formation. Hence it is becoming a strong contender in the development of innovative cell and gene therapies. Horizon's Pin-point base editing technology utilizes an RNA aptamer embedded within the sgRNA to enable the recruitment of a cytidine deaminase for highly efficient and precise nucleotide conversion. The resultant modularity of the platform enables a high degree of flexibility that allows fine tuning of critical aspects of editing behavior, including efficiency, window size and position, and nucleotide context preference. Such adaptability of the system is critical to realize the huge potential of base editing for the generation of cell and gene therapies. In this study, we report the development of arrayed and pooled screening platforms to provide comprehensive characterization of editing performance and to assess guide RNA functionality in a high-throughput manner. Firstly, we describe an arrayed screening platform using five different cytidine deaminases and three structurally distinct tracrRNAs, and assess functionality of Pin-point system architecture at 70 guide-specific genomic sites. We demonstrate significant impact upon editing behavior with different deaminases. Secondly, we present a flexible and adaptable pooled screening reporter platform for high-throughput parallel assessment of >65,000 gRNAs, including 7009 guides targeting pathogenic SNVs. We demonstrate the ability of the pooled screening platform to detect editing in a highly reproducible manner. In summary, we present the Pin-point platform as a tunable, modular system that can be readily adapted to address diverse editing requirements.

1067 Deconvolution of Clinical Variance in CAR-T Pharmacology and Response Using a Mathematical Model of T Cell Differentiation Control

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CAR-T cell expansion and persistence varies widely between patients and is predictive of efficacy. To understand the mechanisms underlying this variance, we developed a mathematical description of T cell regulatory control wherein transitions between memory, effector, and exhausted T cell states are coordinately regulated by antigen engagement. The model was trained using clinical data from CAR-T products in different hematological malignancies (Kymriah and Abecma) and identified cell-intrinsic differences in the turnover rate of memory cells and cytotoxic potency of effectors as the primary determinants of exposure and response. These mathematical inferences were confirmed using bulk and single cell RNAseq data, and population exposure and response predictions validated against registrational data from Kymriah and Yescarta. Using a machine-learning workflow we demonstrate that product-intrinsic differences can accurately predict patient outcomes based on pre-infusion transcriptomes, while additional pharmacological variance arises from cellular interactions with patient tumors. We find that transcriptional signatures outperform T cell immunophenotyping as predictive of clinical response for two CD19-targeted CAR-T products in three indications (CLL, ALL, and LBCL). We describe a "CAR T cell response scorecard" that can be referenced to characterize the transcriptomes of post-manufactured products and more reliably predict patient responses. Combined with our pharmacology modelling workflow, these methods will enable a new phase of therapeutic T cell development, including the use of alternate sources such as induced pluripotent stem cells.

1068 Enhanced Gene Editing by Coiled-Coil Recruitment of an Exonuclease to CRISPR/Cas

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The CRISPR/Cas system is a highly potent tool which has revolutionized genome engineering and regulation of gene transcription in various cells and organisms. This gene-editing tool consists of a guide RNA (gRNA) and Cas9 endonuclease. Cas9 catalyzes the formation of double-strand DNA breaks, which are then repaired by different cell mechanisms. Error-prone Non-homologous end joining occurs, resulting in random indel (insertion-deletion) mutations, which can lead to functional gene inactivation by either frameshift or deletions. To achieve greater indel mutations that can lead to bigger gene disruption, CRISPR system can be coexpressed in cells with DNA exonucleases, which cause increased recessions of DNA following DNA breaks. We show that joint action of the CRISPR system with different exonucleases significantly increases the percentage of indel mutations at various targeted genes. Of the different exonucleases tested, the *E.coli*-derived exonuclease III (EXOIII) exhibited the best performance in terms of indel formation. To further improve the rate of indel mutations, Cas9 and EXOIII were brought into the proximity via coiled-coil forming heterodimeric peptides (CCExo). This resulted in increased indel formation compared to the classical CRISPR/Cas system as well as more efficient than cointroduction of non-interacting and genetically fused Cas9-EXOIII. We discovered that not only the percentages of indel were increased also the size of gene deletions were significantly enhanced compared to conventional CRISPR/Cas system. Genome editing rate was augmented when using heterodimeric coiled-coil forming peptides with higher binding affinity. By high through put method, called CIRCLE-seq we have proven that increase in genome modification does not bare any dangers as no additional undesired DNA cleavage was observed. The robustness of CCExo was determined for different genes in various cell lines, as well in human primary cells and in somatic adult cells. Finally we performed a case study for the use of the CCExo system as a potential anti-cancer therapeutic tool. The Philadelphia chromosome, which occurs in leukemic cancer cells, is the result of characteristic the reciprocal genome translocation t(9:22) and is responsible for higher proliferation of tumorous cells. Using the CCExo system, we achieved a higher degree of indel mutations at the translocation site, which resulted in greater killing of cancer cells, thus providing a useful potential anti-cancer therapeutic tool. This was also confirmed *in vivo* by using xenograft animal cancer model. We successfully confirmed that enhanced genome editing tool, CCExo, holds a tremendous potential not only in treating CML cancer but potentially also in other diseases with genetic etiology.

1069 Exploring Innovative Methods for Enhancing Lentiviral Vector Yield and Target Cell Infectivity in CAR-T Cell Therapy Manufacturing

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In recent years, labs and companies have been seeking ways to reduce the cost of manufacturing CAR-T products to increase the accessibility of the CAR-T treatment. Advances in cell processing and manufacturing techniques have improved in recent years. Along with technology improvements, the viral vector lab are continuously exploring novel methods for enhancing the efficiency and the quality of the procedures in the CAR-T manufacturing and it have been shown as successfully achieved. First, our lab has developed several new viral production cell lines and modified traditional transduction method for the viral production and cell modification. By using the newly developed production cell line, the yield of the viral vector can be significantly increased at least 5 folds in unprocessed viral titer comparing with the traditional adhering method and additional, at least, 25% of the production yield comparing with the current market available suspension culture lines. In addition, using the modified cell line, we found that the capacity of the gene insertion size in the testing viral transfer plasmid can be significant increased into 6~8 KB CDS length while maintaining its related production titer. It provides more flexibility in the vector design. Our lab has also developed a method that enables improved the infectability of cells using a significantly lower viral MOI during the CAR-T development process. With the designed drug treatment, treated cells can be sensitized for infection with 3 folds lower MOI and with relatively higher expression of the genes of interested. This improvement allows the manufactory can produce CAR-T cells in a most cost-effective way. Meanwhile, through the collaborating efforts, we are currently developing new platforms for generating save tools for CAR-T development by using CRISPR technology and the self-inactivated retro-viral vector. This collaboration will focus on the improvement in the Safety and efficiency of the procedures. Combining our several improvements, the result shows our method can be applied into current CAR-T production procedure and is with a significant impact to the operational timing and cost. In addition, our results are also suggested that the on-current procedure may be still with a lot of potential to be improved.

1070 OmniCAR - A Universal CAR T Cell Therapy to Target Multiple Antigens in Blood and Solid Tumours

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Background: Chimeric antigen receptor (CAR) T cell therapy has shown remarkable efficacy in treating blood cancers. However, conventional CAR T cell therapy has thus far shown limited efficacy in solid tumours, due to immunosuppression in the tumour microenvironment (TME), inefficient T cell trafficking, antigen heterogeneity etc. Relapse is common due to tumour escape. **Rationale and Aim:** Therefore, there is an unmet need for a new class of universal immune receptor (UIR) expressing CAR T cells which have the flexibility to target more than one tumor antigen and can be controlled with predictable dose-response kinetics. We demonstrate durable OmniCAR expression and 'arming' with targeting ligands against multiple antigens, with highly specific and inducible, potent activation and anti-tumor function against multiple blood and solid tumours. **Methods:** The OmniCAR UIR is comprised of two components, (i) a cell surface receptor expressed on T cells that consists of the SpyCatcher component and CD3ζ and 4-1BB intracellular signalling domains

and (ii) targeting ligands conjugated to an adaptor peptide sequence (SpyTag). When combined, the targeting ligand and OmniCAR form a covalent bond thereby arming the T cells. **Results:** In vitro co-culture with tumor cells demonstrated titratable dose-responses (0-1000nM binders) and potent anti-tumor cytokine production (IFN, TNF, IL-2) and killing against multiple solid and blood cancer cell lines. Furthermore, we demonstrated in mixed tumor cocultures, strict OmniCAR T cell antigen specificity, with anti-tumor killing elicited only against tumor cells expressing the corresponding target antigen. Sequentially adding a second targeting ligand can redirect OmniCAR T cells to target the tumor cells that initially escaped CAR T killing. In a murine model of HER2+ breast cancer, human OmniCAR T cells proliferated and engrafted without initial 'arming'. Potent therapeutic efficacy was only observed when the anti-HER2 targeting ligand was administered, leading to regression of large tumors. **Conclusion:** UIR are a rapidly emerging arm of adoptive immunotherapy, and here we have demonstrated the potential of the OmniCAR platform to address these challenges through increased safety and reduced side effects (switching on/off CAR responses in vitro and in vivo) and targeting multiple tumour antigens simultaneously or sequentially to overcome escape mechanisms such as antigen loss or antigen heterogeneity.

1071 AAV-ARSA Mediated Gene Replacement for the Treatment of Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is an autosomal recessive neurodegenerative disorder caused by mutations in the arylsulfatase A (*ARSA*) gene, resulting in lower sulfatase activity and the toxic accumulation of sulfatide in the central and peripheral nervous system. In MLD patients, this leads to progressive demyelination, cerebral atrophy, peripheral neuropathy, and shortened life expectancy. Therapeutic benefit of ARSA replacement has been demonstrated in a clinical setting and the sustained expression of ARSA by gene therapy is a promising strategy with potential to restore myelinogenesis, prevent additional atrophy and provide long lasting therapeutic benefit from a one-time treatment. We present an AAV-mediated gene replacement strategy to treat MLD and report therapeutic benefit in a MLD mouse model, and meaningful ARSA expression and biodistribution in non-human primates (NHPs). We report the nomination of a novel capsid AAV.GMU1, demonstrating better biodistribution and higher transgene expression in the CNS of NHPs, compared to a gold-standard CNS-tropic capsid. We demonstrate that AAV.GMU1-h*ARSA* mediated gene replacement in MLD mice (*Arsa*$^{-/-}$) resulted in the reversal of MLD-associated pathology. AAV.GMU1-h*ARSA* treated mice show increased sulfatase activity in the brain and spinal cord and a concomitant reduction in sulfatide levels (LC-MS) in the brain, spinal cord, CSF, and plasma. Treated MLD mice exhibit prominent and persistent h*ARSA* expression (measured up to 13 months post-dosing), secretion, and cross-correction. Furthermore, *Arsa*$^{-/-}$ mice treated with AAV.GMU1-h*ARSA* showed near complete absence of MLD-associated histopathology and absence of MLD-associated phenotypes. Further, a dose-range finding pharmacology and safety study was carried out in juvenile cynomolgus monkeys, that received four increasing doses of AAV.GMU1-h*ARSA*. We report widespread and dose-dependent increase in AAV.GMU1-h*ARSA* vector biodistribution in NHP brain, with clinically meaningful h*ARSA* expression in >90% of the brain. NHPs showed uniform h*ARSA* expression in the spinal cord and DRGs along the spinal rostral-caudal axis, and presented no clinical signs (functional or behavioral) for the duration of the study. In summary, we propose AAV.GMU1-h*ARSA* mediated gene replacement as a clinically viable approach to achieve broad and therapeutic levels of ARSA in the CNS and PNS.

1072 An Antibody-Based AAV Retargeting Platform for Efficient and Specific *In Vivo* Gene Delivery to the CNS

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Adeno-associated virus (AAV) is a leading viral vector used in gene therapy to treat human diseases. Although AAV has many advantages as a gene therapy vector, some of the limitations to its use for disorders of the central nervous system (CNS) are the inability of most wild-type AAV serotypes (AAV9, AAVrh10) to efficiently cross the blood-brain-barrier (BBB), as well as their tendency to preferentially transduce the liver. Although capsid library-based selection approaches are a powerful method to identify novel capsids with desired properties, the translatability of such capsids between species, particularly for human treatment, remains a challenge. Thus, we sought to develop a species-agnostic, modular AAV retargeting platform that utilizes rational capsid engineering and monoclonal antibodies to redirect viral particles to cross the BBB, while minimizing peripheral tissue transduction. Transferrin Receptor 1 (TfR) is a conserved receptor expressed on the apical surface of brain endothelial cells that is known to facilitate transcytosis of cargo across the BBB. We utilized a protein tagging system to covalently couple TfR antibodies or antibody fragments to surface-exposed variable loops on the viral capsid. We observed that multiple AAV serotypes can be efficiently retargeted to the CNS with anti-TfR antibodies following systemic administration, achieving comparable CNS transduction to wild-type AAV9 at considerably lower doses. We also found that multiple antibody formats, including bivalent antibodies, fragment antigen binding regions (fabs) and single-chain fragment variables (scFvs), can facilitate transcytosis of AAVs across the BBB. The natural tropism of the conjugated virus can be further modified by the introduction of specific mutations into the capsid sequence of the TfR-targeted vectors, which substantially reduce transduction of the liver and other non-CNS tissues without impairing the efficiency of CNS gene transfer. Finally, to select the most efficient, translationally relevant antibody candidates for BBB-crossing in humans, we screened 32 anti-human TfR fabs, using IHC and qRT-PCR to quantify their ability to mediate BBB-crossing and transduction

of the CNS following systemic delivery in mice expressing human TfR. Using this approach, we identified multiple fabs with a range of binding properties that facilitated potent AAV delivery to the brain. Our antibody retargeting platform provides a novel method to enhance delivery of AAV therapies to the CNS by targeting a conserved receptor, while simultaneously reducing off-target tissue transduction to mitigate safety risks. We aim to apply this technology to the development of therapies for diseases of the CNS.

1073 Production of AAV Vectors Using Synthetic, Enzymatically Produced Linear DNA

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Adeno-associated virus (AAV) remains a popular vector for gene therapy. However, AAV manufacture faces several challenges to keep pace with the demand for large scale, high quality batches. The gold standard plasmid triple-transfection presents several issues including availability and cost of GMP grade plasmid, sequence fidelity of the Inverted Terminal Repeats (ITR), and safety concerns over non-specific encapsidations. 4basebio has developed a proprietary, scalable synthesis process for the production of linear closed DNA constructs via its Trueprime™ amplification technology. The hpDNA™ produced is devoid of any bacterial backbone and circumvents cumbersome fermentation processes required for plasmid DNA. The process is size and sequence independent, allowing for large scale production of linear DNA with high yield and purity in less than a week. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins, and excludes bacterial sequences such as antibiotic resistance genes. Here, we compared the production of AAV vectors using hpDNA™ encoding for the typical Adenovirus helper functions, *rep* and *cap* genes, and an expression cassette consisting of AAV2 ITRs and a GFP reporter gene driven by an ubiquitous promoter. Conventional plasmid triple-transfection was used as a control. We achieved equivalency in viral genome titres, Full:Empty ratios and infectivity between the two production methods. We have demonstrated that functional AAV vectors can be produced using hpDNA™, which could greatly accelerate therapeutic development of gene therapy programmes. The technology could overcome the difficulties associated with complex ITR structures required for AAV production, which are inherently difficult to synthesise via bacterial propagation systems. Moreover, the lack of plasmid backbone sequences such as antibiotic resistance genes enhances the safety profile of the AAV product.

1074 Characterization of Pre-Existing Immunity to AAV5

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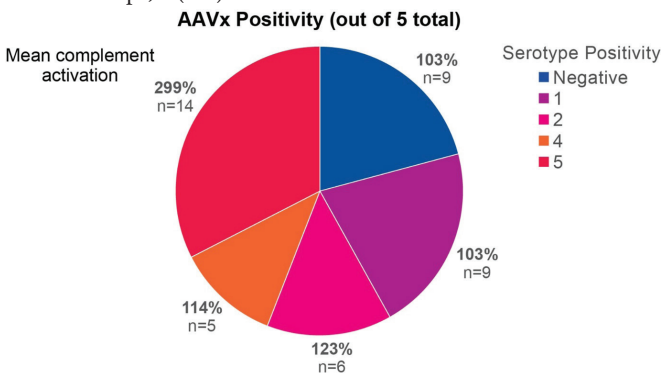
Seroprevalence studies indicate many prospective gene therapy (GT) patients have been previously exposed to adeno-associated viruses (AAV) and harbor pre-existing AAV antibodies, which may impact the administration and efficacy of GT. The objective of this study was to further characterize pre-existing antibody responses to AAV serotype 5 (AAV5) in severe hemophilia A patients (n=540) who are GT naïve. We evaluated AAV5 neutralizing factors (transduction inhibition, TI) using a cell-based assay, AAV5 total binding antibodies (TAb) using a bridging immunoassay, AAV serotype specificity using a bridging immunoassay, AAV5 complement activation, and AAV5 antibody isotypes. Most subjects with pre-existing immunity to AAV5 were positive for both TAb and TI, with a small subset showing discordant results. Pre-existing AAV5 TAb titers were much lower than AAV5 post-treatment titers. We previously[1] assessed the seroprevalence of pre-existing immunity to AAV5 and four additional AAV serotypes (AAV2, AAV6, AAV8, AAVrh10). Using those data, we assigned subjects to AAVx positivity groups (Negative, 1, 2, 3, 4, 5) based on the number of serotypes for which they were positive. There was no association between higher AAV5 TAb titers and the presence of antibodies reactive with other serotypes, suggesting that AAV5-specific TAb titers were not a result of broad previous AAV exposures (and subsequent cross-reactivity). In a subset of subjects (n=43) with and without pre-existing AAV5 TAb positivity, AAV5-induced complement activation was assessed in vitro by measuring complement split products (C3a, C4a, C5a and Bb). In some samples harboring AAV5 TAb, a >200% increase in activated complement split products was observed; however, this was not strictly correlated with the magnitude of AAV5 TAb titers. Complement activation was not observed in TAb-negative subjects, and not all TAb-positive subjects exhibited complement activation. An analysis of data based on AAVx positivity showed that mean complement activation to AAV5>200% in vitro was measured only in subjects that were positive for all five serotypes (AAV5/2/6/8/rh10). Activation of complement may enhance complement receptor-mediated clearance, alter the biodistribution away from the target tissue, and therefore reduce transduction. Finally, in another subset of subjects (n=78) with pre-existing AAV5 TAb positivity, we characterized the isotype and subtype of the anti-AAV5 response (IgM, IgG1, IgG2, IgG3, IgG4, IgA). Responses were predominantly the IgG isotype. Higher AAV5 TAb titers (>100) and TI titers (>60) were typically associated with detection of IgG1, but higher AAV5 TAb titers that were TI negative or low TI titer (<60) did not reveal a similar trend. Consequently, highly neutralizing antibody responses were associated with detection of AAV5-specific IgG1. Our in-depth characterization of pre-existing immunity to AAV5 can help elucidate the most important immunological determinants that limit AAV vectored transduction of target tissues. Understanding these determinants of pre-existing immunity could enable more patients to gain access to GT.

1. Klamroth R, Hayes G, Andreeva T, Gregg K, et al. Global Seroprevalence of Pre-existing Immunity Against AAV5 and Other AAV Serotypes in People with Hemophilia A. Hum Gene Ther. 2022 Apr;33(7-8):432-441.



1075 Gene Editing Repair in *USH2A* Knock-Out hiPSC-Derived Retinal Organoids

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Background: Mutations in the human *USH2A* gene account for most cases of Usher Syndrome type II, a heterogeneous autosomal recessive disorder characterised by progressive retinitis pigmentosa and sensorineural hearing deficiencies. *USH2A* is one of the largest retinal disease-associated genes, with an open reading frame of 15.6 kb whose defects span missense, nonsense, indels, splice site and frame-shift mutations throughout its 72 exons. Mutational hotspots are located in exon 13, where a single nucleotide deletion (c.2299delG) represents the most common pathogenic variant. **Results:** Here, we developed *USH2A* knock-out (KO) human induced pluripotent stem cells (hiPSCs)-derived retinal organoids as a human model of *USH2A* retinal disease. The organoids will be used to investigate different viral gene editing approaches. First, we generated homozygote KO mutations in *USH2A* exon 13 by CRISPR-Cas9 system. We designed a single-stranded oligodeoxynucleotide donor containing a premature stop codon followed by an additional nucleotide to disrupt the *USH2A* open reading frame, which is expected to result into a non-functional Usherin protein. Subclones of these *USH2A* KO hiPSCs have been validated by sequencing, and are being checked for maintenance of pluripotency, differentiation capacity, karyotypic stability and copy number variation analysis, as well as absence of off-target events. The *USH2A* KO hiPSC clones are being differentiated into retinal organoids along with isogenic controls. The potential loss of Usherin protein and *USH2A* transcripts in the *USH2A* KO retinal organoids will be studied. In addition to disrupting *USH2A* exon 13, we are developing retinal organoids with nonsense mutations in exons 60 to 67 of *USH2A*. Previously, we tested transduction of retinal organoids by adeno-associated viral (AAV) vectors which can carry up to 4.5 kb of transgenic DNA. Here we will show transduction of retinal organoids by high-capacity adenoviral vectors (HC-AdV) that can incorporate up to 36 kb of transgene. In addition, we will present our AAV and HC-AdV vectors for Homology-Independent Targeted Integration (HITI) gene editing. **Discussion:** We successfully generated *USH2A* KO retinal organoids and showed efficient transduction of photoreceptors in cultured wild-type retinal organoids by HC-AdV. In future studies, we will fully characterise the *USH2A* organoids and compare the expected ciliary retinal phenotype at different timepoints. We plan to transduce the *USH2A* mutant organoids with AAV HITI *USH2A* editing vectors. However, the entire *USH2A* cDNA sequence is more than three times bigger than the AAV maximum transgene capacity of 4.5 kb. The largest *USH2A* cDNA fragment able to fit in the viral vector extends from exons 2 to 17. Therefore, we designed and constructed a vector incorporating *USH2A* exons 2 through 17 (4 kb) together with the required regulatory elements. This vector system will target *USH2A* intron 17 permitting the testing of a superexon approach for rescuing full-length *USH2A* expression in patient-derived retinal organoids. Double-strand DNA break repair in post-mitotic photoreceptor cells relies on non-homologous end joining (NHEJ). In this work, the DNA knock-in into *USH2A* introns in the retinal organoids will be mediated by HITI. We will examine whether AAV-mediated HITI gene editing will restore the expression of Usherin in the *USH2A* retinal organoids. The retinal organoids with nonsense mutations in *USH2A* exons 60 to 67 will be used to test insertion of 4 kb of *USH2A* cDNA containing exons 60 to 72 plus regulatory sequences into *USH2A* intron 59. We explore in addition the use of HC-AdV vectors for insertion of 15 kb of *USH2A* exons 2 to 72 cDNA and regulatory sequences into intron 1.

1076 A Novel Oncolytic HSV Sensitizes Resistant Glioblastoma Therapy and Anti-Tumor Immunity

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The host immune system has developed multiple mechanisms to defend against and clear virus infections, including PKR, cGAS-STING and TLR-MyD88 signaling. Compromised anti-viral mechanisms in tumor cells allow for infection, replication and lysis by oncolytic virus. However, these compromised antiviral mechanisms, together with immune cells and tumor microenvironment, limit the sensitivity of oncolytic virus in tumor cells. We have generated a novel oncolytic virus oHSV-shPKR which disables tumor intrinsic PKR signaling. oHSV-shPKR results in suppression of innate anti-viral immunity and improves virus spread and tumor cell destruction in both oncolytic viral-resistant and sensitive GBM cells. oHSV-shPKR increases the activation of antigen presentation cells through type I interferon signaling activation and produces an immune-stimulatory function to increase tumor-antigen specific CD8 T cell expansion, including cytotoxic T lymphocytes. Preclinical studies show that intra-tumoral injection of oHSV-shPKR significantly inhibits human GBM PDX tumor growth in immune-deficient NSG mice and murine orthotopic GBM tumor growth in immunocompetent mice. Mechanistic studies using single cell and mRNA sequencing suggest that PKR positively regulates TGF-ß immune suppressive signaling. To our knowledge this is the first report to identify dual and opposing roles of PKR wherein PKR activates anti-virus innate immunity while it inhibits anti-tumor adaptive immune responses. Thus, the novel oHSV-shPKR has the potential to be used in clinical applications for both oHSV-resistant and sensitive GBM treatment.

1077 Mitigating a TDP-43 Proteinopathy Using RNA-Targeting CRISPR Effector Proteins

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are currently incurable neurodegenerative disorders. While each disease affects distinct neuronal regions, ALS and FTD share a highly frequent pathological feature: the aggregation and ubiquitination of the TAR DNA-binding protein 43 (TDP-43). Given its apparent central role in ALS and FTD, TDP-43 has emerged as an important and potentially broadly applicable therapeutic target for both disorders. However, TDP-43 performs countless crucial cellular functions, which thus likely preclude its targeting for sporadic forms of the disorders. As a result, numerous attempts have been undertaken to identify gene targets whose perturbation can modify

TDP-43 toxicity without affecting its expression. One such modifier is ataxin-2, a polyglutamine-containing RNA binding protein that, when modulated, is capable of modifying TDP-43 toxicity without affecting its expression. Thus, strategies for targeting ataxin-2 may hold potential for ALS-FTD. In addition to traditional gene silencing modalities, CRISPR technologies have emerged as broadly effective platforms for perturbing the expression of a target gene, including potentially ataxin-2. Among the CRISPR-based platforms that can be used for this goal are RNA-targeting CRISPR effector proteins, which rely on an engineered CRISPR RNA (crRNA) molecule to recognize a target RNA sequence that facilitates its degradation by the CRISPR effector protein. This ever-expanding toolbox of effector proteins includes the Cas13 family of enzymes, which, after binding to a target RNA sequence, indiscriminately *trans*-cleaves non-target RNAs, as well as Cas7-11, a protein that cleaves its target RNA in *cis* and as such is thought to pose minimal risk for collaterally *trans*-cleaving non-target transcripts. Given their high programmability and capacity to knockdown the expression of target genes in mammalian cells, we hypothesized that RNA-targeting CRISPR effectors could be programmed to target ataxin-2 and consequently, mitigate ALS and FTD-associated TDP-43 pathology. In the present study, we find that RNA-targeting CRISPR effectors can target ataxin-2 to ameliorate a TDP-43 proteinopathy. Specifically, we show that Cas13-mediated ataxin-2 reduction in cell culture models can reduce the size and number of TDP-43 protein inclusions, as well as TDP-43 localization to stress granules, the transient cellular structures that accumulate protein and RNA in response to stress. We further validate the efficiency of our approach by delivering an ataxin-2-targeting Cas13 system to a mouse model of a TDP-43 proteinopathy. Our approach rescued motor impairment, enhanced strength, and dramatically extended survival. Finally, we utilize the ataxin-2 sequence to benchmark the specificity of second-generation high-fidelity Cas13 proteins and Cas7-11, finding that these effectors demonstrate markedly improved capabilities. Our results thus demonstrate that RNA-targeting CRISPR technologies can be programmed to silence disease-modifying proteins and hold potential for TDP-43 proteinopathies.

1078 Preclinical Development of an *Ex Vivo* Gene Therapy for Mucopolysaccharidosis Type II

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Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is an X-linked inherited disorder of carbohydrate metabolism. It is caused by mutations in the *IDS* gene, encoding the lysosomal enzyme iduronate-2-sulfatase (IDS). IDS deficiency causes glycosaminoglycan (GAG) accumulation in the lysosomes, leading to a severe impairment of cell metabolism and finally to cell death. The central nervous system (CNS) is particularly sensitive to this metabolic defect. MPS II causes distinctive facial features, visceral abnormalities and progressive intellectual decline, and the currently available treatment is only symptomatic.We designed an original approach based on hematopoietic stem cell gene therapy (HSC-GT) genetically modified by lentiviral (LV) gene transfer to restore the IDS expression. The approach was tested in vivo in pre-symptomatic and symptomatic MPS II mice. Experimental mice were monitored for survival, behavior and pathology hallmarks long-term. HSC-GT rescued behavioral, skeletal, biochemical and pathological disease-associated deficits and significantly increased animal survival, with all GT-treated mice out-surviving the control mock-transplanted mice. HSC-GT resulted in restoration of IDS activity up to 50% of the normal level and reduction of GAG accumulation in the brain of the treated animals. Safety monitoring of the treated mice did not show any treatment-related adverse events. Based on these results in the animal model of the disease, we assessed the HSC-GT strategy in a toxicology and biodistribution study in humanized immunodeficient mice (NSG). The study employed human HSPCs transduced with large-scale produced Lentiviral vector (LVV) and two different manufacturing protocols intended for clinical use, which differ in the total duration of the LV transduction, cell concentration and cytokine cocktail. Human HSPCs transduced with these protocols showed a preserved functionality in repopulated immunodeficient mice, as compared to mock-transduced cells, with a sustained engraftment up to 16 weeks post-transplant and multilineage differentiation. LVV profiling and *in vitro* immortalization assay revealed the absence of genotoxicity. Interestingly, we observed a robust engraftment *in vivo* of highly-transduced cells with respect to the infused cell products, which is currently being investigated. Overall, the study will be instrumental to a rapid progression toward the clinical development of our HSC-GT strategy for MPS II treatment.

1079 Plenty of Lenti - A Multi-Angle Approach towards Method Optimization of Lentivirus Production

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Gene and cell therapy applications will dominate the biopharma market in the coming years, much like antibodies did in the 2000s. One popular retroviral vector that is already in use for *ex vivo* administration is the lentiviral vector (LV). LV is increasingly used for cell therapy applications as it can transduce a variety of cell types as well as dividing and non-dividing cells. The latter is a game changer for treatment options, as these vectors can also transduce hematopoietic and T-Cells, cell types that have proven difficult to transduce for other viral vectors. However, challenges remain. One that has yet to be overcome is the current demand of LV quantities and required quality for clinical use. Therefore, more efficient production processes for high quality LVs are essential. Here we show how the upstream section of VSV-G enveloped LV production processes in HEK293 cells can be tweaked to help overcome these limitations. In a comprehensive optimization procedure, we tested different transfection protocols and were able to demonstrate the positive effect of adding fresh medium during the LV production process. A stability study for LVs was performed at 37 °C, in which the fragility of the vector during the production process was

investigated. A decrease in infectious viruses below detection limit after 24 h was observed. Based on these results, we compared different harvesting strategies where one single harvest step at the cultivation end versus multiple harvest steps including daily media change were performed. Despite the vector fragility, obtained data demonstrated no advantage in transducing units or total virus quantities when using multiple harvest steps, wherefore a single harvest is more expedient. Shelf life of stored LVs is also an important parameter and was monitored at storage conditions of +4 °C, -20 °C, as well as -80 °C over a period of three month by infectivity assay. Whereas negligible differences in LV infectivity at -20 °C and -80 °C were observed, viral titer at 4 °C showed a 10-fold decrease over time. Another focus of this work aimed at the comparison of the infectivity assay, in an adherent as well as in a suspension-based system. For this purpose, different protocols were investigated in more detail, with the protocol using adherent cells yielding higher transducing units. This work shows the importance of optimizing every step for efficient LV production.

1080 The Role of DNA/RNA Hybrids on the Efficiency of Nuclease-Free Genome Editing and AAV Integration *In Vivo*

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AAV viruses have a natural ability to stimulate homologous recombination (AAV-HR). In addition, rAAV vectors also integrate into numerous locations throughout the genome. We previously demonstrated a promoterless rAAV vector containing host genomic homology arms (e.g.*Albumin*) flanking a therapeutic coding sequence (e.g. human coagulation factor IX) induced homologous recombination in mouse liver (Barzel et al., Nature 2015). This resulted in a chimeric mRNA capable of producing the endogenous albumin and protein of interest from the same transcript. While this approach has led to therapeutic levels of a handful of proteins, the percentage of edited cells remains low (0.2- 1.0%). In order to attempt to enhance the number of AAV-HR positive cells, a genetic screen identified that the inhibition of the Fanconi Anemia complementation group M (FANCM) significantly improve the AAV-HR. FANCM is one of the enzymes required to resolve DNA/RNA hybrids, also known as r-loops, that can occur when a RNA filament anneals to a single strand DNA. The formation of r-loops takes place during DNA transcription and replication, and if not tightly regulated could lead to severe DNA damage such as single and double strand breaks. R-loops can be physiologically resolved by specific helicases such as FANCM or enzymes belonging to the RNase H family which degrade the RNA strand. Here, we report that the genetic inhibition of FANCM and SRSF1, two central proteins involved in r-loop formation, results in a significant 3-fold improvement of AAV-HR in vitro. In addition, using the small molecule topotecan, we demonstrated a significant increase in r-loop production in a murine hepatoma cell line (HEPA1-6) and mouse liver. Topotecan treatment resulted in a up to 15-fold enhancement AAV-HR in vitro and ~3-fold improvement in vivo. We were able to show the mechanism of improved AAV-HR efficiency was indeed due to r-loop formation by blocking the enhancement by co-treatment with RNAseH1, an enzyme required for resolving r-loops. We next elected to map and quantify r-loops throughout the genome. To this end, we performed DNA/RNA immune precipitation sequencing (DRIP-seq) studies in vitro and in vivo. To our knowledge, this represents the first genomic r-loop map of an intact tissue. Notably, we were able to detect massive r-loops accumulation in a specific region of the *Albumin* locus (exons 12-14) where we had designed homology targeting arms (Barzel et al. Nature 2015). We are currently doing comparative genomic studies of r-loop formation in hepatoma cells and intact liver and designing AAV-HR vectors to establish if regions rich in r-loops are predictive hotspots for both nuclease-free and nuclease-dependent AAV-HR. In addition, we also have preliminary data to suggest that regions enriched for r-loop formation are hotspots for "random" AAV vector integration. These findings may shed light on mechanisms helping to improve the safety and efficacy of in vivo genome editing and may enhance our ability to predict regions most susceptible to insertional mutagenesis with canonical rAAV vectors.

1081 Development of a Mass Spectrometry-Based Assay for the Detection of Endogenous and Lentiviral Engineered Enhanced Hemoglobin in Sickle Cells and Mice

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Sickle cell disease (SCD) results from a sequence defect in the adult hemoglobin (HbA) β-globin chain. SCD is traditionally diagnosed by cellulose-acetate hemoglobin (Hb) protein electrophoresis or high-performance liquid chromatography (HPLC). While clinically useful, these methods have sensitivity limitations. In efforts towards implementing a gene therapy approach for SCD, we first developed a novel mass spectrometry (MS)-based method for the rapid, sensitive, and highly quantitative detection of endogenous and lentiviral-encoded therapeutic β-globin. We have engineered a novel lentiviral vector based on the Lenti/βAS3-FB construct. The βAS3-globin transgene expressed from this vector contains the mutations G16D, E22A and T87Q that increase affinity for healthy β-globin and inhibit axial and lateral contacts with sickle β-globin, thereby conferring anti-sickling properties. Utilizing synthetic signature peptides for wild-type human β-globin, sickle β-globin, and βAS3-globin, we developed sample preparation methods and a MS-based assay to simultaneously detect each of these globins in cultured cells and small quantities of mouse peripheral blood. Using these MS methods, we have successfully phenotyped homozygous HbA (AA), heterozygous HbA-HbS (AS), and homozygous HbS (SS) Townes mice. We detected lentiviral vector-encoded βAS3-globin in transduced erythroid cell cultures and in transduced human CD34+ cells after erythroid differentiation. We also detected therapeutic βAS3-globin in peripheral blood six weeks post-transplant of transduced Townes SS bone marrow cells into Townes SS mice. This βAS3-globin persisted over 24 weeks post-transplant. With several genome-editing and gene therapy approaches for severe

hemoglobin disorders currently in clinical trials, this MS detection method will be useful for patient assessment before treatment and during follow-up.

1082 Chimeric Antigen Receptor (CAR) Natural Killer (CAR-NK) Cells for Treatment of COVID Patients

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New severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) variants continue to emerge, the newest variant of concern (VOC) Omicron subvariant XBB.1.5, imposing significant threats to the currently approved vaccines and therapeutics. Immunocompromised individuals are reported to facilitate SARS-CoV2 immune escape mutations throughout the infection course, leading to further SARS-CoV2 evolution. During the first peak of the pandemic, we first proposed to use chimeric antigen receptor (CAR) natural killer (NK) cell-based immunotherapy targeting the spike protein expressed on SARS-CoV2 for the treatment of COVID-19.NK cells provide the first line of defense against viral infections and tumor cells by secreting interferon-gamma to initiate the immune response. NK cells mediate antibody-dependent cell-mediated cytotoxicity by binding to the fragment crystallizable region of a neutralizing antibody (NAb) or mRNA-vaccine-induced antibody to trigger NK cell responses against SARS-CoV2-infected targets. However, SARS-CoV2 directly evades NK cell surveillance by downregulating NKG2D ligands, by which NKG2D is one of the major activating receptors. Moreover, clinical data showed that circulating NK cells are significantly reduced, dysfunctional, and exhibit an exhausted phenotype in severe COVID-19 patients. Altogether, these data indicate the important role of NK cells in controlling COVID-19 disease.Recent studies in CAR-NK cells showed promising results in treating lymphoid tumors with reduced toxicity, including graft versus host disease and cytokine release syndrome compared to that of CAR-T cells in clinical trials. However, one of the major challenges of using CAR-NK cells includes the ability to expand adequate non-exhaustive NK cells. Our lab developed a method to easily generate long-lasting 'memory-like' CAR-NK cells from peripheral and cord blood cells. We, therefore, designed autocrine expressing interleukin 15 (IL-15) third-generation CAR-NK cells expressing S309 single-chain variable fragment (henceforth S309-IL15-CAR-NK cells), where S309 NAb is demonstrated to neutralize both SARS-CoVs by binding to a highly conserved receptor binding domain region with high affinity. By challenging human angiotensin converting enzyme 2 NOD Scid gamma mice with USA/WA1/2020 followed by S309-IL15-CAR-NK cell or PBS treatments, we show that S309-IL15-CAR-NK cells significantly reduce the viral loads of SARS-CoV2 in the lungs of the treated group. We demonstrate efficient binding of S309-IL15-CAR-NK cells to SARS-CoV2 wildtype pseudotyped virus and its VOCs, including the B.1.617.2 (Delta), B.1.621 (Mu), and B.1.1.529 (Omicron). We also show that S309-IL15-CAR-NK cells effectively kill target cells expressing spike mutations derived from SARS-CoV2 variants *in vitro*. Our data collectively suggest the potential use of S309-CAR-NK cells for treating COVID-19 patients, especially those unresponsive to currently available therapeutics due to the emerging VOCs.

1083 Off-the-Shelf Third-Party HSC-Engineered iNKT Cells for Ameliorating GvHD While Preserving GvL Effect in the Treatment of Blood Cancers

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Allo-HSCT is a curative therapy for hematologic malignancies owing to GvL effect mediated by alloreactive T cells; however, the same T cells also mediate GvHD, a severe side effect limiting the widespread application of allo-HSCT in clinics. Invariant natural killer T (iNKT) cells can ameliorate GvHD while preserving GvL effect, but the clinical application of these cells is restricted by their scarcity. Here, we report the successful generation of third-party HSC-engineered human iNKT (3rdHSC-iNKT) cells using a method combining HSC gene engineering and in vitro HSC differentiation. The 3rdHSC-iNKT cells closely resembled the CD4 CD8 /+ subsets of endogenous human iNKT cells in phenotype and functionality. These cells displayed potent anti-GvHD functions by eliminating antigen- presenting myeloid cells in vitro and in xenograft models without negatively impacting tumor eradication by allogeneic T cells in preclinical models of lymphoma and leukemia, supporting 3rdHSC-iNKT cells as a promising off-the-shelf cell therapy candidate for GvHD prophylaxis.

1084 CRISPR-Activated Biomedical Reporter Pigs for *In Vivo* Genome Editing Pre-Clinical Studies

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Translating promising gene editing technologies such as CRISPR-Cas and base editor from basic research tools to therapeutics that cure genetic diseases require relevant preclinical testing to ensure the safety and efficacy. Preclinical studies in large animal models will provide critical data on dosing and safety of both the editor and the delivery vehicle prior to human use. Genetically engineered swine are beneficial as a preclinical model due to their similar size, anatomy, and physiology to humans as well as their <4 months gestation time and high fecundity (>6 per litter). Technology to produce gene edited swine has rapidly advanced, and custom-engineered swine that harbor the targeted therapeutic site from humans in their genome can now be made and delivered in less than 7 months. These animals are particularly useful for late-stage IND-enabling studies like dose-escalation, pharmacokinetics and pharmacodynamics analyses to streamline preclinical evaluation for gene editing-based therapies. In addition, these engineered animals can elucidate which organs, tissues, and cells will be targeted by the delivery vehicle in a large animal and allow for toxicity studies to be performed. One way of measuring the specificity of *in vivo* gene editing in specific organs, tissues, or cells is by using reporter lines, like those pioneered by the rodent community, including the Ai9 reporter. We have developed a biomedical research pig line with a modified Ai9 reporter at the *ROSA26* safe-harbor locus in pigs as part of the NIH Somatic Cell Genome Editing consortium. The modified Ai9 line includes a human Ubiquitin C or CAG promoter driving a tdTomato fluorescent reporter that is activated after DSB-

induced removal of a series of transcriptional stop signals (Figure 1). After activation, analysis can be performed in organs, tissues, and cells using immunohistochemistry, single cells using fluorescence activated cell sorting, molecular analysis, or direct sequencing (Figure 2-4). In addition to the fluorescent protein, the reporter will express a sodium iodide symporter (NIS) that will allow reporter activation to be detected non-invasively by PET or SPECT imaging for longitudinal studies. This model is available with a ~4 month lead time, and pre-clinical experiments can be performed at Recombinetics or through a contract research organization.

Figure 1: Modified Swine Ai9 reporter

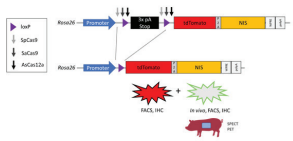


Figure 2: FACS analysis of Ai9 fibroblasts

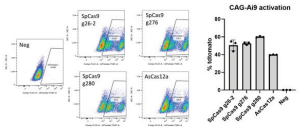


Figure 3: IHC analysis in activated reporter liver tissue

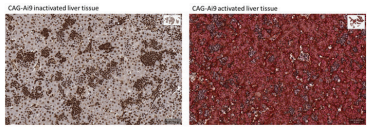
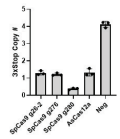


Figure 4: Droplet digital PCR analysis of pig Ai9 fibroblasts



1085 *Ex Vivo* and *In Silico* Modelling of Human Innate and Adaptive Immune Responses to AAV Vectors

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Despite clinical successes achieved with recombinant Adeno Associated Virus (AAV) therapeutics, host immune responses against the vector have been observed in numerous clinical studies. Understanding of these responses is limited by the high variability among individual subjects, insufficient or low sample size in rare disease gene therapy trials, the limited access to relevant clinical samples, and the lack of animal models that would be predictive of the cytotoxic immune response observed in humans. Given this gap in current knowledge, we have exploited human *ex vivo* systems previously validated for predicting immunogenicity of biological drugs, to identify assays reliably predicting AAV vector immunogenicity. Primarily, innate immune responses to AAV were mapped by measuring vector-induced pro-inflammatory cytokine release from the whole blood (WB) or the PBMC fraction isolated from healthy donors. Both methods showed that the increase in interleukin-6 had the highest magnitude across all tested cytokines and was the most frequent response among all tested donors (n=40), with the whole blood assay being more sensitive compared to the PBMC assay. Next, we showed that WB assay can be used to demonstrate differences in the immunogenicity of different AAV serotypes and manufacturing methods, or distinct innate activation patterns of DNA cargos packaged into identical capsid serotypes. Further, we used *in silico* analysis (EpiMatrix software) to confirm the relatively low immunogenicity of the AAV capsid protein compared to other viral epitopes or immunogenic antibodies. The presentation of AAV epitopes by human antigen presenting cells was further verified using the MHC-associated peptide proteomics (MAPPs) assay. The AAV epitope presentation was functionally verified by the DC:T cell proliferation assay performed using PBMC samples obtained from new set of healthy donors (n=40). We showed efficient proliferation of $CD4^+$ T cells, with only a minority of donors triggering $CD8^+$ T cell proliferation. In addition, a Fluorospot assay was used to further characterize AAV-specific $CD4^+$ T cells isolated from a subset of donors (n=20), which showed production of IFNγ and TNFα cytokines. Finally, using the above immune assay data, we adapted the Immunogenicity Simulator, a computational platform, to model a virtual AAV clinical trial (ranging from 40 to 500 virtual subjects). Identical to what was observed in real-life AAV clinical trials, modeling predicted the AAV dose- and time- dependent increase in anti-AAV antibodies and a correlation between the loss of AAV-transduced hepatocytes and the expansion of a pool of effector $CD8^+$ T cells. This work provides insights into the mechanisms driving human immune response to AAV vectors and lays the foundation for future correlations of immunogenicity predictions with clinical outcomes in gene therapy trials.

1086 Nanoplasmid™ Vector Platform for Superior AAV and mRNA Vector Yield and Stability

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The Nanoplasmid™ vector backbone (<500 bp) is a replacement for previous generation bacterial backbones that improves safety and performance of gene and cell therapeutics. Nanoplasmid vectors do not include antibiotic resistance markers which improves safety compared to plasmids or minicircles that rely on antibiotic resistance for bacterial selection. Nanoplasmid vectors also contain a specialized bacterial R6K replication origin in place of the traditional pUC replication origin, making the Nanoplasmid vectors replication-incompatible with native organisms—this is an additional safety factor that reduces Horizonal Gene Transfer risk, since Nanoplasmids™ can only replicate within the specialized *E. coli* host strain used for manufacturing. Two of the leading biotechnology platforms, Adeno-associated virus (AAV) gene therapy vectors and mRNA vaccines, encode structured DNA elements essential for their function. AAV transfer plasmids contain highly structured palindromic Inverted Terminal Repeats (ITRs) flanking the 'payload' transcription unit. These ITR repeats, which contain the viral replication origin and viral packaging signals, are highly unstable and difficult to replicate in Escherichia coli, creating challenges for AAV transfer plasmid manufacture. mRNA vaccines are manufactured from polyA -repeat containing template plasmid vectors that contain an antigen gene downstream of a bacterial promoter, and upstream of a polyA repeat. To manufacture the mRNA vaccine, the template vector is produced in *E. coli*, then linearized by restriction digestion downstream of the polyA repeat. A mRNA encoding the antigen gene with a stabilizing polyA tail is then produced by *in vitro* runoff transcription. Similarly to ITR repeats, the long (80-120 bp is

typical) polyA repeats are often prone to deletions and the vectors are often low yielding in Escherichia coli. To stabilize the structured DNA repeats in AAV and mRNA vectors, the typical approach is to use recombination deficient hosts such as SURE or Stbl4. While these strains can be used to stabilize and produce AAV and mRNA vectors in small scale shake flask culture, the multiple mutations characterizing these strains lead to very poor biomass and vector yields in the large-scale fermentation required for production of vectors for clinical and commercial applications. The REVIVER host strain, an engineered cell line that improves stability and replication of structured DNA sequences while retaining high fermentation productivity, is a superior high yielding *E. coli* host for manufacture of AAV transfer plasmids and polyA repeat containing mRNA template plasmids. Fermentation yields and stability are often lower with AAV transfer plasmid and polyA repeat containing mRNA template plasmids than standard plasmids. This appears to be due to the plasmid backbone encoded pUC replication origin having difficulty replicating closely positioned structured DNA sequences. We have developed an alternative R6K replication origin-based vector backbone, the Nanoplasmid backbone, which has dramatically improved fermentation manufacturing yields with these structured DNA containing vectors. Nanoplasmid retrofits of existing AAV transfer vectors or mRNA vaccine vectors stabilize and improve DNA vector manufacture, while not changing the sequence or composition of the vector payload (AAV virus or mRNA transcription unit in these cases).

1087 A Six-Day Enhanced Clinical Scale Manufacturing of Transduced T-cells for Immunotherapies

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Chimeric antigen receptor (CAR) -modified T-cell therapy against CD19 has emerged as a significant therapy against relapsed/refractory lymphomas. However, patients typically require a high dose of T-cell infusion (~2 × 106 to 2× 108 T-cells/kg) for treatment while the manufacturing time to generate consistent CAR T-products cells is a lengthy process ranging from 8-14 days. Furthermore, quiescent, and naïve T cells are recalcitrant to the fusion of lentiviral particles. It is therefore necessary to either activate naïve T cells prior to transduction or to employ a high multiplicity of infection (MOI) of the lentiviral vectors (LVs) to overcome the limitation of hard to transduced primary T cells. Vectofusin-1, a soluble short cationic peptide is used to improve transduction efficient of murine CAR T cells. However, it was shown to exhibit lesser CAR-T transduction efficiency in comparison to RetroNectin. We have recently identified another novel type of soluble additive that exhibit similar T-cell transduction efficiencies as mediated by RetroNectin, and the goal of the current study is to determine the efficiency of T-cells transduction and expansion using this agent in an in-house optimized cell manufacturing process. In the latter, T cells were activated using soluble CD3/CD28 antibodies in a previously selected serum free media, denoted SFM#3, and spinoculation was performed together with the new additive using a second generation humanized CD19-CAR (CV -hCD19). As a reference, similar number of human peripheral blood mononuclear cells (2.7 x 10^8) from the same healthy donor were processed using the standard T-cell transduction (TCT) protocol whereby the cells were activated with TransAct while spinoculation was performed in the presence of vectofusin-1. Using the CliniMACS Prodigy platform, the results showed that 59.1% of T cells were positively transduced by CV-hCD19 at a MOI of 5 with a low vector integrant copy number less than 2. In contrast, 44.1% of CD19-CAR T cells were obtained using the same MOI with the standard TCT protocol. At day 6 where CD19-CAR T cells were harvested, the total number of CD3+ cells had increased from 49.3% to 91% (or 89% as in TCT protocol). Interestingly, CD19-CAR T cell fold expansion in our process was demonstrated to be double that of standard TCT protocol with no notable increase in exhaustion markers as compared to the non-transduced T cells. Moreover, we observed an improved fraction of self-renewing multipotent memory T-cells (Tscm+Tcm= >8%) in the final product using our method. The final CD19-CAR T-cells products from both methods were subsequently subjected to a co-culture cell kill assay using NALM-6-Luc tumor cells stably expressing luciferase. An augmented elimination of CD19 tumor cells (>20%) was also observed from cells using SFM#3 consistent with the higher transduction efficiency when compared to standard TCT. In conclusion, we were able to generate CD19 CAR+ T-cells with better CAR expression and efficacy, in a short six-day manufacturing process with final product attributes analogous to clinical formulations. An accelerated improved expansion protocol for CAR T-cells manufacturing would reduce the cost of goods and improve patient access to CAR-T cell therapies for increasing patient benefit.

1088 A Novel Homogenous Bioluminescent Immunoassay to Quantitate AAV Capsid Titer and Manufacturing-Related Protein Impurities

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Adeno-associated viruses (AAVs) have gained significant attention in the field of gene therapy due to their capacity to effectively deliver therapeutic genes into human cells. Recombinant AAVs (rAAV) are produced primarily in mammalian cell culture. Despite extensive viral purification processes, removal of protein impurities has been difficult. Current methods to assess AAV capsid titer and protein impurities present challenges including low sensitivity, high cost, and laborious workflow. We have developed a homogenous (no-wash) plate-based immunoassay technology that can accurately quantitate rAAV viral capsid titer and detect residual protein contaminants from rAAV manufacturing. The Lumit™ immunoassay platform is based on NanoLuc® Binary Technology (NanoBiT®), which is a structural complementation reporter composed of two subunits: Large BiT (LgBiT) and Small BiT (SmBiT). Antibodies against the analyte of interest are labeled with either LgBiT or SmBiT. Upon binding to the analyte, the antibodies bring the SmBiT and the LgBiT subunits into close proximity, resulting in the reconstitution of a functional luciferase. Consequently, luminescent signal production is directly proportional to the amount of analyte. To develop Lumit™ AAV immunoassays, antibodies against specific serotypes were selected and labeled with SmBiT or LgBiT. Standard curves of rAAV2, rAAV8 and rAAV9 generated with our Lumit™ immunoassays displayed outstanding sensitivity, with up to 3 orders of magnitude in dynamic range. Lumit™ immunoassays can tolerate a variety of sample matrices including cell

lysate, viral lysis buffer, and purification buffers. Furthermore, during AAV manufacturing, there are several common sources of protein impurities, including host cell proteins, benzonases, and human serum albumin. We have also developed Lumit™ immunoassays for each of these impurities, all of which showed significantly improved sensitivity at limit of detection compared to the respective ELISA assays. Overall, our data show that the Lumit™ immunoassay is a powerful alternative to conventional ELISAs for quantifying rAAV viral capsids and detecting protein impurities during rAAV manufacturing. Lumit™ immunoassays allow for rapid analyte quantitation with simple, sensitive, and reproducible workflows.

1089 Optimizing PSCA CAR T Cells with Bispecific T Cell Engagers to Target Two Distinct Subtypes of mCRPC

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Metastatic castration resistant prostate cancer (mCRPC) comprises of two distinct sub-types, prostate adenocarcinoma (PrAd) and neuroendocrine prostate cancer (NEPC). Treating advanced mCRPC remains a significant challenge effective treatment strategies are needed. Current targeted therapies often result in transient anti-tumor responses and often lead to antigen-negative tumor outgrowth. Chimeric antigen receptor (CAR) T cell therapies are being actively investigated to target mCRPC, including CARs targeting prostate stem cell antigen (PSCA), which is overexpressed in PrAd. However, it is anticipated that for durable antitumor responses, NEPC-targeting will be required. Carcinoembryonic antigen (CEA) is overexpressed in NEPC and may be effectively targeted with T cell engagers. Here, we outline the development of mouse PSCA-CAR (mCAR) T cell therapy in combination with a CEA bispecific to target mCRPC in a clinically relevant syngeneic tumor model. This model was developed to express human CEA and PSCA allowing us to evaluate safety of this dual targeting strategy. Initial *in vitro* studies interrogated the efficacy of PSCA mCAR T cells loaded with a murine dual bivalent CD3xCEA bispecific T cell engager (CEA dbBiTE). We found that although antigen-specific cell lysis was observed, the presence of dbBiTE on the surface of T cells was short lived. To address this, we optimized manufacturing and loading of mCARs. Current studies are underway to optimize the treatment strategy of PSCA mCAR T cells loaded with CEA dbBiTE *in vivo*. Future work will evaluate the *in vivo* safety and efficacy of PSCA mCAR T cells engineered to co-target CEA for the treatment of advanced heterogeneous mCRPC.

1090 Establishing a Platform Process to Manufacture the Highest Quality Precision Plasmids

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Since the first pharmaceutical application of plasmids (their introduction into bacteria to enable the production of therapeutic proteins) was achieved in the 1970s, plasmids have become critical starting materials for many advanced drug products. They are used to transfect mammalian cells to manufacture complex recombinant proteins, antibodies, and other biologic drug substances. With the increasing investment in advanced therapies such as viral vectors, cell-mediated, and nucleic acid-based modalities, plasmid manufacturing has become a major bottleneck to deliver these therapies. Plasmid transfection into mammalian cells is the predominant production method for viral vectors. Plasmids also serve as the template for mRNA manufacturing and have potential applications as vaccines and other therapeutics. Establishing a platform plasmid manufacturing process that is robust, scalable, and generates high quality pDNA that meet stringent regulatory requirements is necessary to support advanced therapy manufacturing. To develop such a manufacturing platform requires in-depth knowledge of the upstream, mid-stream, and downstream unit operations (specifically when different E. coli strains and a range of plasmids are used). A design-of-experiment (DoE) approach was taken to characterize processes and determine critical processparameters (CPPs) that impact critical quality attributes (CQAs) for E. coli fermentation and downstream plasmid purification. Subsequent data will highlight critical process parameters and their impact on both yield andquality. To develop a flexible platform, the upstream DoE approach explored different plasmid characteristics (size, complexity, and origin of replication) and multiple E. coli strains. Four different plasmid sequenceswere investigated. Three plasmids representing commonly used sequences for the production of recombinant AAV vectors, including pRepCap, pHelper, and pGOI (gene of interest) were chosen. The pGOI constructrepresents the complex secondary ITR sequence that can be difficult to properly amplify due to its complex repeating structure. The fourth plasmid was a green fluorescent protein (GFP) reporter plasmid with longterminal repeat (LTR) sequences for lentiviral (LV) vector production. This case study will highlight a flexible platform for plasmid DNA manufacturing

1091 CRISPR Based Site-Directed RNA Editing in Patient iPSC-Derived Neuronal Models of Genetically-Linked Intellectual Disabilities

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Pathogenic G-to-A mutations that manifest as neurological disorders have traditionally been challenging to edit in neuronal cells as current strategies require host-cell DNA repair pathways and incorporation of a template sequence. ADAR2 is a brain-enriched protein which post-transcriptionally modifies double-stranded RNA (dsRNA) by deaminating adenosine nucleotides to inosine, thereby enabling translational machinery to create a functional adenosine to guanosine edit within the transcript. The CRISPR associated protein 13 (dCas13b) is a catalytically inactive RNA binding protein which can achieve site-directed RNA editing when fused to the ADAR2 deaminase domain (ADAR2DD). A guide RNA (gRNA) sequence forms a dsRNA substrate for dCas13b-ADARDD2 binding at the complementary target while enabling RNA editing at a single base. In addition, designing the gRNA with a cytosine nucleotide opposing the target adenosine further specifies ADAR2DD activity within the target transcript. However, the CRISPR Cas13 system has yet to be employed for RNA editing of endogenous G-to-A mutations in neuronal cells. Our lab created induced pluripotent stem cell (iPSC) from fibroblasts of individuals with a pathogenic G-to-A mutation in exon 5 (E198K) of protein

phosphatase 2, regulatory subunit B', delta (*PPP2R5D)* which are causative for the neurodevelopmental disorder Jordan's Syndrome. Here, we have differentiated isogenic and variant iPSCs to neural stem cells (NSCs) to evaluate RNA editing efficiency and efficacy. Towards this goal, we have designed and screened exon 5 (E198K) gRNA to identify lead gRNA which have high on-target editing and low-to-moderate off-target editing in the *PPP2R5D* transcript. We demonstrate gRNA length and A-C mismatch position as important factors for on-target editing efficiency. We have further expanded our disease model by differentiating isogenic and E198K NSCs to neurons to understand the neurodevelopmental consequence of G-to-A mutations on *PPP2R5D* targets at the phosphoproteomic and transcriptomic levels. In addition, these studies have provided insight into potential pathways that are candidates for molecular rescues with our CRISPR RNA editing approach. Our future work is focused on employing the CRISPR RNA editor system in these patient-specific neuronal models. These studies support the potential for the CRISPR/dCas13b system to selectively edit mutant transcripts harboring G-to-A mutations in neuronal cells while providing an alternative editing technology for neurodevelopmental disorders.

1092 The VeCap® Platform: Structurally Engineered Tissue Specific AAV Capsids

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Recombinant Adeno Associated Virus (rAAV) vectors are the leading gene delivery tool for human gene therapy because of their advantageous safety profile and potential long-lived therapeutic effect. However, the broad tissue tropism and the high vector dose required to sufficiently transduce the target organ are still limiting factors for their clinical use. At VectorY, we have established a modular platform aimed at generating VeCaps®, Next Generation tissue specific rAAV capsids. We have designed a rational approach to engineer the rAAV capsid utilizing protein structural engineering to enable capsid binding to a specific cellular membrane protein (or receptor). Target membrane proteins are identified via defined selection criteria and novel peptides are designed to bind these proteins, followed by structural integration of the peptides to the viral surface. Via the structural engineered specific peptides, these novel VeCaps® convey specific tropism and improved transduction efficiency. VeCaps® are produced in a baculovirus/insect cell-based manufacturing process, resulting in high yields of rAAV vectors with a scalability advantage. The VeCaps® are screened and selected in a panel of human primary and iPSC-derived, 2D and 3D, *ex vivo* models relevant to Central Nervous System, and validated in large animals for biodistribution. Our platform provides the opportunity to explore capsid engineering in conjunction with functional data, to accelerate the rational design of AAV capsids that will advance VectorY's vectorized antibody programs. Through this approach, we, in a short time, identified multiple VeCap® variants that show up to 100-fold improved transduction, when compared to the commonly used HEK-produced AAV9. Work is ongoing to characterize the biodistribution of the lead candidates in Non-Human-Primates. In conclusion, the VeCap® platform provides a rapid capsid development platform with high success rate to generate potent and tissue-specific rAAVs compatible with industrial scale processes.

1093 Targeting FVIII Expression to Liver Endothelium

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Hepatocytes have long been considered the FVIII producer cells and thus key target cells for HA cell and gene therapy. However, recent studies have demonstrated that FVIII is largely synthesized and secreted by endothelial cells (ECs), and specifically by liver sinusoidal endothelial cells (LSECs). LSECs represent the main population of non-parenchymal cells in the liver and the major source of FVIII in the body, therefore they represent a suitable target for gene therapeutic approaches. Among the different functions that belong to LSECs, one is their high endocytic activity through clathrin-dependent endocytosis mediated by scavenger receptors and resulting in the clearance of waste from the bloodstream. Stabilin-2 (STAB2) is a scavenger receptor and is involved in the binding, uptake and degradation of multiple ligands such as hyaluronic acid and factor-FVIII complex. Our group previously demonstrated in a murine HA model that transduction of LSECs with a LV carrying the FVIII transgene under the control of an endothelial promoter is able to correct the bleeding phenotype for long term with no inhibitor formation. Since STAB2 is normally expressed by LSECs, we investigated whether the STAB2 promoter could be used to direct specific *in vivo* expression in LSECs upon LVs transgene delivery, thus resulting in a stable expression of the therapeutic protein due to the presence of natural regulating elements within cells and to the tolerance induction against the delivered transgene. To these aims, we *in vivo* delivered in mice by LV injections two transgenes driven by the STAB2 promoter: GFP, as a reporter gene for investigating the cell-specific expression and the tolerance induction; FVIII for testing the therapeutic correction of the hemophilic phenotype. To investigate the role of STAB2 promoter in modulating *in vivo* the immune response in the liver of LV-injected mice, we employed the Jedi mice which present a high percentage of GFP-specific TCR CD8 T cells. LV.STAB2-GFP and LV.PGK-GFP have been injected in B10D2 mice, syngeneic with Jedi mice, and at day 10 CD8 T cells isolated from B10D2 (control) or from Jedi mice have been adoptively transferred into B10D2 mice. GFP expression was analyzed 14 days after LV delivery by IF of livers. GFP expression driven by STAB2 promoter was visible in all the conditions, with no differences between mice that received only the vector or CD8 T cell injection. In particular, despite the use of Jedi CD8 T cells, which specifically detect the transgene (the epitope of the GFP expressed by MHC II), GFP was clearly present in endothelial cells, recognized by their typical shape in contrast with F4/80 staining lining the macrophages. To study whether endothelial-specific FVIII production would support long-term transgene expression in a different immunocompetent mouse strain, we injected HA mice with both LV.STAB2-hFVIII. Again, no plasma anti-FVIII antibodies were found in both vectors used (leading FVIII), with detected activity of the transgene in plasma maintained between 10 and 14 % respectively over time. The activity was sustained until 52 weeks, the longest time tested,

confirming that correction was achieved in all injected mice. These final data reinforce our hypothesis that endothelial-specific expression driven by STAB2 promoter leads to stable FVIII expression and activity without the formation of neutralizing antibodies in immunoreactive HA mouse strains, confirming the power of this novel promoter.

1094 AAV Gene Therapy for TNNT1 Nemaline Myopathy

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TNNT1 Nemaline Myopathy is a fatal congenital disease caused by mutations in the TNNT1 gene encoding for the slow skeletal troponin T1 subunit in the sarcomere. The troponin T1 subunit links the troponin complex to tropomyosin, forming the troponin-tropomyosin complex. Numerous recessive genetic mutations, as well as one dominant mutation, affecting the TNNT1 gene have been described. The first and most extensively described mutation is the c.505G>T homozygous nonsense founder mutation resulting in a premature stop codon in exon 11, affecting the Old Order Amish population of Pennsylvania, USA. The Amish mutation results in the loss of tropomyosin-binding site 2, and all recessive mutations described thus far affect the C-terminal region of the troponin T1 protein. The Amish mutation is characterized by progressive muscle weakness and pectus carinatum, resulting in hypoventilation and fatal respiratory insufficiency around 24 months of age. Like other nemaline myopathies, histology of muscle tissue of TNNT1 Nemaline Myopathy patients shows rod-like aggregates in the sarcoplasm. In our experience, a published TNNT1 knock-out mouse model displayed no overt phenotype and unexpectedly, western blot analysis detected TNNT1 protein in knock-out mice. Considering these challenges, we generated a new TNNT1 knock-out model with a deletion of exons 2 through 14 using CRISPR/Cas9. As of 8 weeks of age, no overt phenotype has been detected. To fully characterize this new model, treadmill endurance testing, rotarod, plethysmography (respiratory) testing, and western blot analysis are underway. Currently, the new TNNT1 KO mouse colony is fully expanded and new AAV gene therapy experiments are being initiated. We developed several vectors encoding human TNNT1 with expression driven by different muscle specific promoters for packaging in AAV8 and AAV-MYO2A. Additionally, we obtained embryos of a natural TNNT1 Nemaline Myopathy merino sheep model from Australia which we intend to use as a large animal model for future therapeutic development.

1095 circAde, a Vector System for Spliceosome Dependent circRNA Biogenesis and Prolonged More Effective Protein Expression

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Circular RNA (circRNA) constitutes a novel class of endogenously expressed RNA. In contrast to mRNA, circRNAs are resistant to exo-nucleolytic decay which results in high intra-cellular stability and persistence. Understanding of circRNA production, both *in vitro* and *in vivo*, has improved significantly in recent years, and it is now feasible to engineer circRNAs for highly efficient protein translation. As such, engineered circRNAs are emerging as a novel therapeutic concept that may overcome several challenges facing mRNA. Production of therapeutic circRNAs can be achieved by two distinct mechanisms, either 1) using group-I-intron derived ribozymatic circularization for *in vitro* production, or 2) using spliceosome-based backsplicing similar to endogenous circRNA biogenesis for *in vivo* production. In the latter setup, inserting flanking inverted repeat (IR) elements is known to stimulate backsplicing dramatically by positioning the splice sites involved in close spatial proximity. Here, we show that our proprietary circRNA expression system, circAde, allows high yield circRNA production through spliceosome-dependent biogenesis. Using a panel of proof-of-concept-reporters, we show that certain design-features greatly influence circRNA production and subsequent protein expression, in particular choice of IR and IRES (internal ribosome entry sequence) elements. Publicly available RNA sequencing datasets were interrogated to identify IR elements flanking the most abundant circRNAs across multiple cell types. Candidate IRs were tested in our circAde system, and those capable of stimulating the highest level circRNA production, were modified to further enhance circRNA yield. Devoid of a 5'cap, circRNAs rely on IRESs for effective translation. Surprisingly, in addition to facilitating circRNA translation, we observe that choice of IRES also impacted circRNA biogenesis and yield. By conducting two orthogonal IRES screens on 1000+ putative IRES elements, we have identified the most effective IRES sequences in melanoma and lung-cancer cell lines supporting effective circRNA production and high-yield protein expression. Furthermore, comparing protein output from circAde and mRNA-based vectors, we show that the circAde-system out-performs conventional mRNA-based approaches, both in terms of transgene yield and temporal sustainability of expression. In addition, the circAde-system is highly versatile when it comes selection of both transgene payload and vector backbone. In summary, our results demonstrate technical proof-of-concept for a new platform for development of novel, more effective strategies for the treatment of diseases requiring durable transgene expression.

1096 Precision-Targeted Epigenome Editing Enhances CAR T Functional Profiles and Anti-Tumor Activity

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Cell-based immunotherapy with gene-modified T cells with a synthetic Chimeric Antigen Receptor (CAR T) has become a successful clinical treatment paradigm for hematological malignancies, though effectiveness in solid tumors has been elusive. Clinical experience in the heme setting has helped to define some mechanisms of disease relapse with initial generation of CAR T cells, revealing important roles for cellular persistence and functional potency for mediating durable clinical responses. In the solid tumor setting, achieving clinical success has proven to be more difficult, with functional persistence of CAR T cells being hampered by sustained antigen exposure and suppressive features of the tumor microenvironment (TME) driving T cell functional exhaustion. Current preclinical data demonstrating enhanced CAR T cell activity - either by transgenic gene overexpression or gene knock-out(s) to modulate molecular pathways - suggest that engineering of T cells beyond CAR may have beneficial clinical effects that could overcome treatment hurdles in solid tumor settings. However, these approaches result in permanent or non-physiological molecular alterations of cells that may not be ideal for the inherent biology or plasticity of T cells. Additionally, it is likely that modulation of multiple molecular networks is necessary to overcome biological barriers needed to achieve clinical benefit against solid tumors, presenting a significant safety risk for engineering approaches that rely on breaking the DNA strand. Targeted repression or activation of individual genes has been shown using modified gene-editing molecules such as enzymatically-dead Cas9 (dCas) linked to protein domains that activate or inhibit gene transcription via recruitment molecular complexes, without physical disruption of the DNA sequence. Here we show for the first time transiently-delivered dCas epi editing constructs mediating activation or repression of key target genes for improving CAR T cell function. Electroporation of T cells with dCas-epi editor mRNA and gRNAs during CAR T cell production results in temporary expression of the targeted epi editor molecule, followed by a durable modulation of target genes, which is maintained through cryopreservation and functional assays, both in vitro and in vivo. CAR T cells treated with the epi editor and target-specific guide RNA demonstrated markedly improved attributes associated with functional persistence, including elevated expression of key effector cytokines (IL2, IFNg, TNF), enhanced proliferation, and sustained serial target cell killing. Using a subcutaneous xenograft model of Her2-expressing human non-small cell lung cancer cell line NCI-H1975, Her2-specific human CAR T cells treated with dCas-epi editor mRNA and gRNAs exhibited superior tumor control and survival of engrafted mice, with enhanced pharmacokinetics of the CAR T cells in the blood. We furthermore demonstrated that the transiently-delivered dCas-epi editor mRNA and gRNAs can be multiplexed to achieve compounded impacts on functional qualities of the CAR T cells. These results demonstrate a novel approach to safely and effectively epigenetically modulate T cells in a precisely targeted manner to achieve improved outcomes in clinically relevant models of CAR T cell function.

1097 An Artificial Intelligence Approach to Extract Domain-Specific Insights from over 5,000 ASGCT Conference Abstracts

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Natural Language Processing (NLP) is a subfield of Artificial Intelligence (AI) that has been used to process large corpus of natural language data to enable human interpretation. Here we build a gene therapy-specific NLP platform to parse through over 5,000 abstracts from the past 5 years of ASGCT Annual Meetings and extract domain specific topics. Briefly, the abstracts were parsed and represented as numerical vectors via embeddings in a biomedical domain specific language model. The abstracts were then clustered by similarity, and top occurring words per cluster were derived, essentially representing topics captured in the similar abstracts. This Semantic Topic Modeling approach leverages context captured in the dense embeddings. Expert curation of derived topics helped refine data-derived topics relevant to gene therapy domain. We were thus able to represent the whole corpus of abstracts in distinct specific topics based on unbiased text analysis, rather than category of abstract submission. Our platform can be used for exploration of large gene-therapy specific collections of text data, such as articles from PubMed or conference proceedings. The platform can be extended to derive a gene-therapy specific knowledge graph to facilitate discovery of emerging relationships between concepts, or to track trends in the field by quantifying changes in representation of various topics over time. We expect that this application would be of interest to researchers trying to isolate topic-specific information from domain-specific large publications datasets.

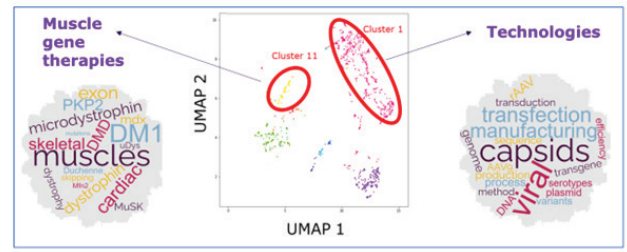

Figure 1: Exploring 2022 ASGCT Annual Meeting themes: abstracts are clustered and most frequently occurring words elucidate the topic of the cluster. For example, inspecting Cluster 1, we see that capsid engineering and manufacturing are the leading themes of the Technologies cluster.

1098 AAV-NRIP Gene Therapy Rescues Motor Functions in Amyotrophic Lateral Sclerosis Model Mice

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Nuclear receptor interaction protein (NRIP) is calcium/calmodulin binding protein involved in muscle functions. Both global NRIP

knockout mice (gKO) and muscle-restricted NRIP knockout (NRIP cKO) show muscular weakness. The interaction between motor neuron (MN) and muscles is mutual regulation for muscle contraction. Intriguingly, loss of muscle NRIP can retrogradely cause motor neuron degeneration in mouse model; indicating muscle NRIP can retrogradely control motor neuron growth. Additionally, NRIP can directly interact with acetylcholine receptor (AChR) as a component of neuron muscular junction (NMJ) formation. Hence, NRIP is a novel AChR binding protein to stabilize NMJ. The abnormal NMJ is a major cause of motor neuron degeneration (MND). The disease of amyotrophic lateral sclerosis (ALS; is one of the MNDs) show progressive neurodegeneration and abnormalities at the NMJ. Until now, there is not yet a cure treatment in ALS. Due to NRIP functions for NMJ maintenance, the role of NRIP in $hSOD^{G93A}$ mice (ALS mouse model) would be interesting to explore; and it may be able to develop NRIP as a therapeutic agent for ALS. Our preliminary results showed that the protein expression of NRIP in spinal cord, gastrocnemius (GAS) and tibialis anterior (TA) muscles were downregulated in $hSOD^{G93A}$ mice compared with WT mice. AAV-NRIP gene therapy in $hSOD^{G93A}$ mice was performed to evaluate the efficacy of AAV-NRIP in $hSOD^{G93A}$ mice. The results revealed the forced NRIP expression by AAV in SOD^{G93A} mice partially rescued motor neuron number, increased NMJ formation, enhanced axon innervation along with increased compound membrane action potential (CMAP) and motor functions. In sum, AAV-NRIP can be a potential drug for ALS in the future.

1099 coAAV Vectors from the AAV-Ligand Conjugate (ALIGATER™) Platform Outperform AAV9 to Address Neurological Disorders via CSF Delivery

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Adeno-associated virus (AAV) is the vector of choice for *in vivo* gene therapy. One of its most exciting advantages lies in its versatility in terms of applications and routes of administration (RoA). Consequently, the AAV platform has evolved quickly over the past decade, moving from the historical natural capsids to hundreds of new synthetic variants, all with their own set of transduction properties. The technology however still faces many challenges and improving features such as vector spreading from the delivery site, AAV transduction and transgene expression, or the immune profiles of AAVs still concentrates a lot of efforts worldwide. Many are focused on modifying the capsid at a genomic level to meddle with epitopes involved in cell tropism. Such genetic mutations, however, can destabilize AAV formation, negatively affecting downstream processes and production titers. And in any case, such genetic variants warrant the development of a dedicated CMC process, which can considerably slow the translation of a therapeutic vector to the clinic. The AAV-Ligand Conjugate (ALIGATER™) platform is a robust alternative to capsid genetic engineering, which relies on the chemical conjugation of small, functionalizing ligands at the surface of the AAV capsid. As it requires no prior genetic intervention and is performed on AAV post production, this platform can be applied easily to virtually any natural or engineered AAV serotype. Here, we demonstrate that ALIGATER™ is particularly exciting for applications in the central nervous system (CNS). Building on our initial results with conjugated AAV (coAAV) vectors, showing improved brain distribution and transduction following intra parenchymal delivery in both rodents and non-human primates (NHP), we investigated the performance of coAAVs in the brain when administered directly into the cerebrospinal fluid (CSF) - a safer delivery procedure than the one tested before. To this end, we built a library of coAAVs and performed a parallel evaluation of these vectors with AAV9 in the NHP brain following intra-cisterna magna (ICM) delivery. Whereas AAV9 is considered as one of the best-in-class serotypes to effectively transduce the brain via this RoA, some serious adverse events have been reported following its use in both primate studies and clinical trials. Safety concerns were largely linked to dorsal root ganglion (DRG) and liver toxicities, resulting from elevated transduction and transgene expression levels in organs. In this study, we showed that at equivalent doses, several coAAVs presented biodistribution and transduction profiles comparable to AAV9 in the brain, while displaying low levels of transduction and transgene expression in the DRGs and most peripheral tissues. These results thus demonstrate that ALIGATER™ is a safe and efficient platform for neurological applications. It can be used to generate strong and robust proof of concept studies in small animals with good translatability to larger animals, accelerating the development of safe therapeutic vectors for neurological disorders.

1100 Development of De Novo Mining Platform for Discovery and Identification of Novel Small Class II CRISPR Nucleases with Expanded PAM Preference

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Clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) system holds great promise to revolutionize genome editing and therapy in basic and translational settings. Various CRISPR-Cas systems have been explored and engineered, such as CRISPR-Cas9 and CRISPR-Cas12a that belong to the Class 2 of CRISPR-Cas system. However, *in vivo* applications of these tools remain limited in certain delivery methods due to their relatively large payload size, PAM restrictions and potential off-target effects. To address these challenges, we mined an extensive genome-resolved metagenomics database and identified an array of novel CRISPR effectors (300 to 700 amino acids) from natural environments. We streamlined the pipeline of predicting and identifying crRNA and tracrRNA from CRISPR operon by RuvC domain-based Hidden Markov Models and small RNA-seq, respectively. We also developed a robust screening method in mammalian cells for rapid identification of the recognizable PAM of each Cas effector. Our platform uncovered dozens of novel Cas nucleases with diverse PAM preferences. We selected one representative Cas (referred as GEBxII305) and engineered both the protein and single guide RNA based on the modeled R-loop complex structure. The engineered GEBxII305 shown a non-T-rich PAM preference and the on-target editing potency was dramatically

improved by up to 10 folds across 20 loci in human cell lines, HEK293T and HepG2. We combined the engineered GEBxII305 with optimized nuclear localization sequence (NLS) to further increase the genome editing efficiency to nearly 20 folds compared to the original version. Off-target profiling of the engineered GEBxII305 by GUIDE-Seq showed comparable levels of off-target effects in human genome with those well-documented CRISPR-Cas nucleases. We further applied the engineered GEBxII305 to primary cells, such as primary human hepatocytes and T cells, demonstrating its robust editing efficiencies. Taken together, we show that these novel Cas nucleases discovered by our platform are potent, specific, and have translational potentials for broad use in cell and gene therapy development.

1101 Intelligent Phage-Assembled Gene Expression (iPhAGE) System: A Novel Platform for Safe Nonviral Gene Therapy

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Small molecules of DNA known as plasmids are found naturally in bacteria. Engineered plasmids can be powerful tools to transfer genes between different organisms. However, plasmids carry elements necessary for replicating in bacteria, which are superfluous and even detrimental for gene transfer or gene therapy applications in humans or animals. Plasmid backbone prokaryotic DNA encoding antibiotic resistance genes, origins of replication and other genetic elements and sequences recognized by the mammalian immune system can interfere with the expression, redose-ability and durability of the transferred gene. The phage M13 is a simple virus that specifically infects the bacterium *Escherichia coli*. Since phages like M13 cannot infect nor replicate in animal cells, they have immense potential as safe vectors for ferrying DNA cargo of virtually unlimited capacity in humans and have even been shown to cross the blood brain barrier. We examined how M13 can be used to improve plasmid vectors for gene therapy by generating "miniphagemids" lacking the problematic bacterial DNA. We constructed a plasmid that split up phage replication signals such that a gene of interest (GOI) cistron cassette could be inserted between them. Replication by M13 then produced virus particles only carrying the genes of interest without the rest of the plasmid. Despite the modifications, miniphagemid yield was comparable, if not superior, to that of wild-type "wildtype" phagemids with unmodified, intact replication regions. Hence, we demonstrated an elegantly simple approach for highly efficient production of gene delivery minivectors using phage M13. To improve the efficiency of miniphagemid production in absence of contaminating helper phage we further produced a novel and highly effective helper plasmid that fully complemented necessary M13 replication and structural components without any active contaminating plasmid packaging in presence or absence of the phagemid construct. We then characterized the capacity of miniphagemid particles for gene transfer into mammalian cell lines in vitro. As a targeting proof of principle, phage display of epidermal growth factor (EGF) was fused to pIII and enabled targeting of cells that overexpress the EGF receptor. Hybrid EGF-displaying phagemids encoding the *luc* reporter gene were transfected across $EGFR^+$ and $EGFR^-$ mammalian cell lines. Gene expression was assessed by examining resultant luciferase activity. EGF-displaying miniphagemids improved gene expression up to three times over full-length phagemids across different types of human cells suggesting the potential for an in vivo targeted gene delivery application of these miniphagemids. In conclusion, we demonstrated that phagemid miniaturization improves gene expression in human cells without detrimental impact on vector production. We also showed that phagemid miniaturization can be combined with phage display, a powerful technique to modify phage properties, such as enabling it to target human cells. This 'intelligent Phage-Assembled Gene Expression' (iPhAGE) system was not impaired in production by the separation of f1 *ori* domains and, in fact, led to superior rescue of miniphagemids by the helper plasmid and iPhAGE vectors also enhanced phage-mediated gene delivery in receptor-specific manner. Overall, we present here a novel targeted gene delivery platform based on the bacterial virus M13, showcasing its immense flexibility as a therapeutic platform. This technology is currently being extended to also generate linear covalently closed dsDNA targeted miniphagemids.

1102 Non-Human Primate Pharmacology of AAV Gene Therapy Vectors for Hereditary Angioedema

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Adeno-associated viruses (AAVs) show promise as gene delivery vectors for genetic diseases including hemophilia A and B, Duchenne muscular dystrophy, and spinal muscular atrophy. Hereditary angioedema (HAE) is a rare genetic disorder characterized by swelling attacks that occur in cutaneous and submucosal tissues. HAE types 1 and 2 are caused by mutations in *SERPING1*, which codes for C1-INH protein. Deficiency of functional C1-INH, a serpin superfamily protease inhibitor, results in the production of excess bradykinin, the biological mediator of swelling. Liver is a major source for the production of C1-INH protein, and therefore a gene therapy approach in which AAV transduction of hepatocytes results in *SERPING1* transgene expression and secretion of C1-INH protein into the circulation appears feasible. A major challenge to this therapeutic approach is the very high levels of serum C1-INH anticipated to be required for therapeutic benefit. Modeling work from C1-INH replacement therapies provides an estimate of the expression level required to render 85% of patients attack free. While achieving such levels of secreted protein using AAV has proven feasible in mice, species differences in vector tropism have made it challenging in more clinically-relevant species such as non-human primates. To

evaluate feasibility of a gene therapy as a therapeutic approach in HAE patients, AAV vectors designed to produce therapeutic levels of serum C1-INH expression at clinically-relevant dose levels were generated and evaluated in non-human primates (*Macaca fascicularis*). One-time intravenous doses resulted in serum C1-INH levels in NHPs above the predicted clinically-efficacious C1-INH level and above the normal human C1-INH level, suggesting that AAV vectors may have promise for gene therapy of HAE types 1 and 2.

1103 Efficient Transgene Knock-In in Human iPS Cells Combined with Small Molecule-Mediated "On-Switch" Yields Clonal Populations of Engineered Tissue-Specific Cells

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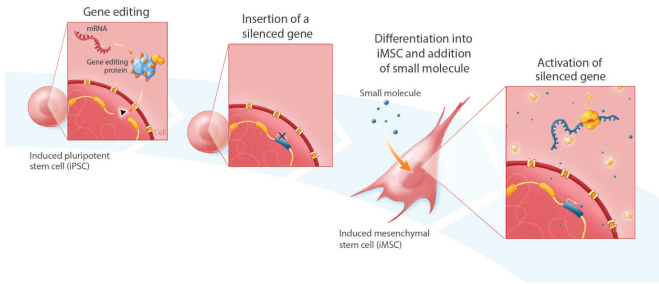
Mesenchymal stem cells (MSCs) are a promising cell-therapy platform with the potential to treat a diverse array of diseases due to their immunomodulator properties - properties which can be enhanced through gene editing. Gene editing autologous or donor-derived MSCs is challenging due to the non-clonal nature of these cell sources, and associated risks of off-target effects. In contrast, iPS cells, which are clonal and highly expandible, provide an ideal source of cells for gene editing and subsequent differentiation into tissue-specific cells for cell therapy applications. Here we establish stable clonal human induced pluripotent stem (iPS) cell lines engineered to express green fluorescent protein (GFP) using two different promoters. We then differentiate the engineered iPS cells to MSCs (EiMSCs) while monitoring changes in GFP expression, isolate an EiMSC subpopulation and verify differentiation using surface markers. Single-stranded DNA (ssDNA) donors encoding GFP under the control of the JeT or EF1α promoters were inserted into iPS cells using mRNA encoding UltraSlice gene-editing proteins targeting the AAVS1 safe-harbor locus. GFP transgene insertion rates of 40% and 10% were observed for JeT and EF1α promoter-containing donors, respectively. While EF1α and JeT promoters drive robust GFP expression when inserted directly in iMSCs, in iPS cells strong GFP expression was only observed under EF1α - expression was not detected in JeT-GFP iPS cells. Clonal cell lines were generated from both lines using single-cell deposition, and bi-allelic insertion into the AAVS1 locus was verified by amplicon sequencing. Engineered iPS cells were differentiated into EiMSCs, and GFP expression was monitored. During differentiation, the number of GFP-expressing cells decreased from >99% in the starting EF1α-GFP iPS cells to 40% in the differentiated EF1α-GFP iMSCs. In contrast, JeT-GFP iPS cells began expressing GFP during differentiation, but stopped expressing GFP near the end of the differentiation process. Treatment with Trichostatin A, a selective histone deacetylase (HDAC) inhibitor, resulted in a temporary increase in GFP expression in JeT-GFP cells during differentiation. To obtain a clonal population of EiMSCs that uniformly express GFP, we then enriched the GFP-expressing EF1α-GFP iMSCs, resulting in a cell line that exhibited traditional MSC surface markers (positive markers: CD90, CD73, CD105, and CD44; negative markers:CD34, TRA-1-60, TRA-1-81, CD45, and HLA-DR) and displayed stable GFP expression for over 7 passages. Here we demonstrate a platform for developing clonal EiMSC cell populations that uniformly and stably express a desired protein from a transgene inserted into a defined genomic locus by mRNA gene editing. We also show temporal control of transgene expression using small molecules during directed differentiation of iPS cells. This platform benefits from high knock-in efficiency enabled by mRNA gene editing combined with ssDNA donors and may prove useful for the development of cell therapies engineered to express therapeutic proteins.



1104 Therapeutic Transgene Levels Can Be Achieved by *In Vivo* Selection of Randomly Integrated rAAVs

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Liver targeted recombinant adeno associated viruses (rAAV) typically have only a short therapeutic benefit when administered to neonatal or pediatric patients. This is due to their predominantly episomal expression that is lost during cell divisions as the child grows. While rAAVs can persist through multiple rounds of cell division by random integration into the genome, these integration events are rare and too infrequent to reach the therapeutic threshold of transgene expression required for successful gene therapy. We hypothesized that in vivo selection of targeted hepatocytes would allow these random integration events to reach a therapeutic threshold. We have previously developed a pharmacologically selectable shRNA that can expand a small population of hepatocytes in the liver with the desired gene edit in vivo. This shRNA functions by blocking expression of Cytochrome p450 reductase (Cypor) which is required for the metabolism of the drug acetaminophen to a hepatotoxic byproduct. Accumulation of this hepatotoxic byproduct leads to cell death. By creating a loss of Cypor linked to the desired gene edits, therapeutic cells can no longer metabolized acetaminophen and are protected from toxicity when high doses are administered. rAAVs containing a U6-driven shRNA targeting Cypor as well as a CAG promoter driving a human Factor IX (hFIX) transgene was delivered into wildtype neonatal and adult mice. Mice were then treated with acetaminophen. Immunofluorescent staining of the liver for Cypor shows large areas of clonal expansion of Cypor-negative hepatocytes, demonstrating successful selection of hepatocytes containing rAAV integrations. Because the rAAV construct expresses the shRNA in cis with hFIX, we would expect these Cypor-negative hepatocytes to also be hFIX positive. Interestingly, we observe both hFIX positive and negative clonal expansions among the Cypor negative hepatocytes. This suggests a rearrangement of the rAAV either during

rAAV production or integration of the vector into the hepatocytes. The composition and location of these rearrangements and their integrations will be further assessed by long read sequencing, which will serve to elucidate the nature of random rAAV genomic integrations. As a control for the randomly integrating vector, selectable vectors with arms of homology directing integration to one of two rAAV integration hotspots (the Albumin gene or the 28S ribosomal RNA locus) were also created. Mice treated with these vectors and subsequent selection will also be analyzed, and the proportion of on target to random integrations will provide additional insight on the integration preferences of rAAV vectors.

1105 Generating a Sheep Model and Developing a Gene Therapy for Sialidosis

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Sialidosis is a lysosomal storage disease caused by mutations in the *NEU1* gene that encodes the lysosomal sialidase neuraminidase 1 (NEU1). Deficiency of NEU1 causes accumulation of sialylated glycopeptides and oligosaccharides in tissues and body fluids, resulting in cell and organ dysfunction. Sialidosis has two forms the late onset Type I or early onset Type II. Normosomatic, Type I patients have adolescent onset with action myoclonus, progressive ataxia, seizures, cherry-red spot, and progressive loss of vision. Symptoms of Type II infantile/juvenile patients are more severe, including hepatosplenomegaly, coarse facial features, dysostosis multiplex, developmental delay, and a short life expectancy. Currently, there are no approved therapies for sialidosis. Our team has developed an AAV-mediated gene therapy strategy to treat sialidosis in small and large animal models. We have developed three AAV9 vectors encoding NEU1, alone or with cathepsin A (CTSA), and either encoding native NEU1 or with modifications to enhance stabilization and secretion. Initial testing of intravenous injections in wild type (WT) mice showed significantly increased NEU1 expression and enzymatic activity ranging from 4 to 40-fold over control mice in the liver and kidney, showing proof of concept and safety. Preliminary results of neonatal intracerebroventricular injections in NEU1 knockout (KO) mice show a higher body weight at weaning age, (n=12) when treated with either of the three vectors (14g- 15g, SD<1.5) compared to untreated mice (11g, n=3, SD=1). Additionally, increased weight of treated NEU1-KO mice is comparable to treated (n=19) and normal control mice (n=10) (15-17g, SD<1.5). Future experiments including behavioral testing to assess memory, cognition, and motor abilities, enzyme assays, biodistribution, and histology will also help determine the most efficacious vector design. The safest and most efficacious vector will be further optimized using a large animal model of sialidosis that we generated using CRISPR-Cas9 technology. Early-stage sheep embryos were electroporated with SpCas9 and RNA guides from exon 5 and 6 of *NEU1*. Genome edited embryos resulted in lambs with NEU1 activity ranging from 0-56% of normal and a phenotype associated with Type II sialidosis, presentation includes head tilt, nystagmus, vacuolated distended neurons, cerebral hemorrhage, hepatosplenomegaly, severe spine curvature, bone deformities, bladder dysfunction, and blindness. To minimize neonatal lethality, we selected a sheep with a Type I patient-like mutation (A319V) and expect homozygous affected sheep in late spring. We have also used double-stranded DNA (dsDNA) donor templates with our current CRISPR-SpCas9 strategy to generate humanized point mutations for Type I (P316S) and Type II (F260Y) sialidosis in additional sheep models. Experiments using dsDNA templates in sheep embryos produced ~80% dsDNA template insertion efficiency, and embryos with the dsDNA template will be implanted in mid-February 2023. Altogether, these studies in parallel with natural history studies in sialidosis patients will inform future clinical trials for this devastating disease.

1106 AAV Serotype Difference in Dose-Dependent Modes of Entry to Cardiomyocytes

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AAV9 (clade F), AAVrh.10 (clade E) and AAV6 (clade A) have each been shown *in vitro* and *in vivo* to have tropism for cardiomyocytes following intravenous administration. Based on knowledge that the first step of AAV interaction with cells is through binding to a cell surface receptor, while each of these serotypes are cardiotropic, we asked: are there similarities or differences in what these serotypes use to interact with cardiomyocytes? To investigate this, we compared the interaction of AAV9, rh.10 and 6 with 2 human cardiomyocyte cell lines, T0539 and AC16. At each of the same doses ($2x10^3$, $2x10^4$ and $2x10^5$ genome copies/cell), AAV6-mediated expression of the β-galactosidase marker gene was 10-fold higher than AAV9 or rh.10, suggesting that AAV6 may use a different mode of entry to cardiomyocytes than AAV9 or rh.10. Based on prior studies of cell populations other than cardiomyocytes, we focused on the interaction of each serotype with receptors AAVR (KIAA0319L), the type I transmembrane receptor used by most AAV serotypes, and sialic acid, identified as a primary or coreceptor for some serotypes. The contribution of AAVR to AAV infection in cardiomyocytes was assessed by (1) blocking of AAVR using an anti-AAVR monoclonal antibody; and (2) upregulation of AAVR by transfection with a plasmid expressing an AAVR cDNA. In the anti-AAVR blocking experiments, anti-AAVR was able to reduce gene transfer by AAV9 and AAVrh.10 to T0539 or AC16 cells by at least 90% ($p<0.05$ for both comparisons). In contrast, anti-AAVR did not cause a significant decrease in AAV6 gene delivery to either T0539 cells or AC16 cells ($p>0.1$). In receptor upregulation experiments, exogenous expression of AAVR more than doubled βgal expression mediated by

both AAV9 and AAVrh.10 in T0539 and AC16 cells ($p<0.01$ for both comparisons). In contrast, exogenous expression of AAVR failed to produce a significant increase in AAV6-mediated gene expression in both cell lines. To assess sialic acid dependence, T0539 cells and AC16 cells treated with neuraminidase to remove sialic acid led to a reduction in AAV6-mediated βgal expression in T0539 (93±1%, $p<3x10^{-6}$) and AC16 cells (91±2%, $p<3x10^{-8}$). In contrast, neuraminidase treatment resulted in a significant increase in AAV9- and AAVrh.10-mediated gene expression in both T0539 and AC16 cells ($p<0.01$, for both lines). Consistent with the studies in the cardiac cell lines, the dependence of AAV9, rh.10 and 6 on AAVR was confirmed by comparing infectivity of HeLa cells and HeLa-AAVR-knockout (HeLa-KO) cells. AAV6 infection of HeLa cells showed a dose-dependent increase in βgal expression ranging from $2x10^3$ to $2x10^6$ gc/cell. In contrast, AAV6 infection of HeLa-KO cells showed no expression at the lowest doses ($2x10^3$ and $2x10^4$ gc/cell) but dose-dependent expression was observed at higher doses ($2x10^5$, $2x10^6$ gc/cell). By comparison, AAV9 and AAVrh.10 showed dose-dependent infection of HeLa cells but no βgal expression at any dose in HeLa-KO cells. Regarding sialic acid dependence, following treatment of HeLa cells with neuraminidase, βgal expression mediated by AAV6 was reduced by 92 ± 4.3% ($p<3x10^{-4}$). In summary, while AAV9- and AAVrh.10-mediated transduction of cardiomyocytes is highly dependent on AAVR, AAV6 transduction of cardiomyocytes is highly dependent on cell-surface sialated molecules.

1107 Online Monitoring of Cell Growth and Shape in Transient Transfection Process for AAV Production in Stirred Tank Bioreactor

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While cell and gene therapies (C&GT) are gaining traction in the clinic with some notable successes, the manufacturing process for these therapies remains challenging and costly. The transient production of viral vector used in C&GT, such as Adeno Associated Virus (AAV), is dependent on consistent upstream processes for batch-to-batch reproducibility. Such consistency is made possible with proper access to process parameters like viable and total cell densities. The iLine Pro F (Ovizio) is an automated cell analyzer to monitor cellular dynamics through on-line bioreactor connection. It relies on a combination of quantitative phase imaging for label-free cell identification and feature extraction algorithms for measuring 30+ parameters for each individual cells such as diameter, elongation, membrane regularity. These parameters are then combined through machine learning algorithms to compute total and viable cell densities. The continuous stream of data provided enables better decision-making during transfection and production phases. We evaluated the iLine F Pro as a tool for real-time HEK293 culture monitoring in manufacturing scale bioreactors for an AAV product. For comparison, the cell density was also monitored off-line using the Vi-CELL counter (Beckman). The data was processed to optimize a raw version of Ovizio's algorithm to a final version based on historical data set. A good correlation between cell densities and viabilities was obtained with the iLine F and the Vi-CELL XR for un-transfected and transfected cells. The developed algorithm can be used for new experiments to replicate the Vi-CELL counts in a label-free and on-line manner. The frequent measurements of the iLine F PRO (1 every hour) can be leveraged to predict when the target cell concentration for transfection will be reached. Pilot tests showed promising results on the accuracy of a predicting algorithm to determine target cell concentration for transfection. This represents an interesting approach to optimize planning of transfection reagent mix preparation. After transfection, a new cell population presenting morphological properties that could correspond to transfected cell population was identified. Monitoring of this population would allow access to transfection efficiency in real-time. As a conclusion, we highlighted the high potential for online monitoring of the cell culture as a tool to increase process understanding, crucial during development stage, and as a first step toward process automation.

1108 Optimization of Chitosan-Lipid Hybrid Nanoparticles for mRNA Delivery

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Introduction: Gene therapy based on messenger RNA (mRNA) has emerged as a powerful tool due its high efficiency and wide range of therapeutic applications such as protein replacement, genome editing, vaccines, and immunotherapies. Compared with plasmid DNA (pDNA) for gene therapy, mRNA has several advantages: protein expression is faster because it does not need to cross the nuclear barrier, mRNA synthesis is relatively quick, and mRNA vectors are safer than pDNA vectors as they do not integrate in the host genome avoiding mutagenesis. However, the success of mRNA therapy depends on the design of safe and efficient gene carries that protect it from RNAses and deliver efficiently to target cells. Recently, we synthesized chitosan-lipid hybrid nanoparticles (named chitosomes) with DOPE/DOTAP surfactants and arginine-modified chitosan (CH-Arg) which showed a highly efficient transfection rate with pDNA. Here, we aimed to evaluated physicochemical properties of chitosome-mRNA complexes and efficiency of mRNA delivery. **Methods:** Different chitosomes were prepared by the association of DOPE/DOTAP lipids and CH-Arg by reverse phase evaporation technique, and after these were complexed with mRNA expressing EGFP. Dynamic light scattering (DLS) and zeta potential (ZP) techniques were used for chitosome and chitosome-mRNA characterization, and agarose gel electrophoresis was performed to assess the efficiency of complexation. *In vitro* transfection was assessed by the fluorescent green protein (GFP) expression in HEK293T cells by fluorescence microscopy and flow cytometry, and cytotoxicity was also assessed by flow cytometry. **Results:** At the optimal ratio concentration (8:1 chitosome:mRNA), chitosomes had small size (75.0 ± 3.6 nm), low polydispersity (0.272 ± 0.040) and positive charge (+28.7 ± 2.8 mV) and were able to fully complex with mRNA, as shown by agarose gel electrophoresis and decrease of charge (+12.0 ± 3.1 mV). However, the complexes chitosome-mRNA showed big size (1772.5 ± 236.2 nm) and high polydispersity (0.505 ± 0.293). After 24 hours of transfection the efficiency was 32.9% ± 5.3 but, interestingly, after 48 hours the transfection efficiency increased (65.85% ± 5.31) and showing a good cell viability (69.3% ± 6.5). **Conclusions:** Taken together, the results show that chitosomes are efficient to stably complex with mRNA

and to deliver to HEK293T cells. Further optimization of the chitosome structure and its complexation with mRNA can further increase the mRNA delivery capacity, making the chitosome a promising system for gene therapy applications. **Funding Support:** FAPESP: 2022/04361-7, 2020/06913-1, 2015/20206-8; CNPq: 303646/2019-5

1109 Oncogenic Extrachromosomal DNA Targeting and Detection Using CRISPR Technology

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Metastases account for the great majority of cancer-associated deaths. Intratumoral heterogeneity contributes to cancer drug resistance, so the elucidation of underlying mechanisms is very important for controlling the growth and spread of primary tumors. Extrachromosomal DNA (ecDNA) was early described as "double minute" and recent studies unveiled that ecDNA is primary source of oncogene amplification in tumor cell. ecDNA was found in nearly half of human cancers but it was almost never found in normal cells, which shows that ecDNA is tumor-specific. Integrating ultrastructural imaging, long-range optical mapping and computational analysis of whole-genome sequencing revealed ecDNA contains highly accessible chromatin where ecDNA drives massive oncogene expression. CRISPR is the immune system in bacteria to defend against invading nucleic acids and has been repurposed for DNA and RNA editing. In this study, we will use two CRISPR system (Cas3 and Cas12) for ecDNA targeting and detection, respectively. Generating a spectrum of large genome deletions render Cas3 potential in ecDNA destruction. We first proposed to target ecDNA with Cas3. In addition, Cas12 is used for DNA detection due to the collateral effect. Development of ecDNA detection using cas12 will facilitate cancer diagnosis. Furthermore, autophagy is a catabolic process aimed at recycling cellular components and damaged organelles in response to diverse conditions of stress. Activation of cell autophagy has shown to reduce the dysregulated DNA amplification and inhibit tumor progression. Collectively, exploit of CRISPR system and autophagy activation in ecDNA detection and elimination pave a new avenue for cancer therapy.

1110 Case Study: Development and Scale-Up of a Helper Dependent Adenovirus (HDAd) Process Using the iCELLis Bioreactor Platform

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Viral vectors are currently the preferred gene-delivery vehicle for most cell and gene therapies and clinical trials require high titer virus preparations to adequately deliver the therapeutic transgenes to clinical subjects or target cells. For this reason, technologies that enable the industrialization of these processes in a safe, robust, and cost-effective way are necessary to support the demands of the patient population. However, complexity around developing and scaling viral vector processes to commercial manufacturing scale, and the lack of standardized approaches remain as challenges that can impact therapy development timelines and productivity. Selection of the appropriate production platform plays a key role on the successful implementation of a process that meets the commercialization timelines and manufacturing costs. We will share a case study that demonstrates the successful steps taken to develop and scale up a customer process for a Helper-Dependent-Adenovirus (HDAd), leveraging Pall's process expertise, and the iCELLis* bioreactor platform, to help accelerate the development timelines. Critical process parameters for development and manufacturing, such as seeding density, infection density, and harvest strategy, were first tested at the flatware stage, and continually optimized over the course of the iCELLis Nano development stage. The optimized parameters were used successfully in duplicate scale-up batches at the iCELLis 500+ scale to demonstrate a process ready for transfer to a clinical manufacturing facility. *registered trademark

1111 Placental-Derived Mesenchymal-Like Adherent Stromal Cell (MLASC) Therapy Results in Alterations in Gene and Protein Signatures Associated with Inflammation and Fistula Formation in Patients with Crohn's Disease

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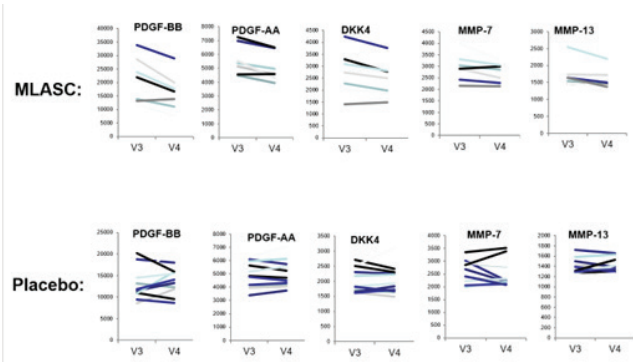
Background: Crohn's disease (CD) is a chronic inflammatory bowel disease. The treatment goal is to control inflammation, induce clinical remission and reduce the incidence of fistulae. Fistulae, caused by epithelial-to-mesenchymal transition (EMT), occur in up to 50% of patients. Transforming growth factor-beta (TGF-β) is an important inducer of EMT by regulating loss of epithelial and gain of mesenchymal markers. Clinical remission rates of 50% at 2 years were observed in 3 early phase studies of IV MLASC therapy in moderate-to-severe CD. To understand the mechanisms of response to MLASC therapy, gene expression and proteomics data was generated to detect clinical response signatures, identify mild/severe CD pathways, and identify changes associated with fistula formation. **Methods:** 2 groups evaluated: MLASC responders (N=12) and controls (N=12) at 3 time points: visit 3 (baseline), visit 4 (early post-treatment) and visit 7 (later post-treatment). Patients were profiled from peripheral blood using transcriptomics/proteomics platforms. Transcriptomics data characterized gene expression in cells of whole blood; Proteomics data characterized protein in plasma. To understand significance of expression changes, pathways enriched in the differentially expressed genes (DEGs) and proteins (DEPs) were identified. Specific DEGs and DEPs that are known to be related to fistula formation were measured.**Results**: MLASC treatment resulted in change in gene and protein signatures trending to gene upregulation and protein down regulation. Numbers of genes/proteins were found to be significantly associated with clinical response (table 1). At visit 4, genes upregulated in MLASC vs placebo were responsible for cell cycle/proliferation and immune response. By visit 7, down-regulation of immune response and inflammation-related genes was observed in MLASC patients, suggesting a therapeutic effect. 113 DEPs were significantly correlated with disease severity. DEGs associated with mild disease were related to immune response and inflammatory markers - primarily to interferon-mediated immune response. Considering fistula formation, increased DEGs expression responsible for immune cell signaling and growth

factor signaling (HGF, PI3K and AKT) was observed. DEGs decreased were responsible for TGF-β, Th2 T-cell response and TNFα signaling suggesting a transition from a fistulizing state. Between visit 3 and 4, in the MLASC patients, repression of plasma proteome markers associated with EMT were observed (Figure 1). **Conclusion:** In CD, MLASCs resulted in alterations in gene and protein signatures associated with inflammation and fistula formation. This analysis provides insight into pathways associated with severity of CD and suggests that gene and protein profiling of blood plasma might be useful for assessing disease severity and treatment response and also suggests that MLASC's may have potential in limiting fistula formation and warrants further evaluation.



	Timepoint	Direction of gene/protein regulation	Transcriptomics	Proteomics
Mean Expression vs Control	*Visit 4*	Down	50	291
		Up	173	1
	Visit 7	Down	59	177
		Up	100	0
	Visit 4 and 7 pooled	Down	41	285
		Up	119	1
Correlation with time	*Linear stream visits 3-7*	Down	3	13
		Up	1	43

1112 Method Development and Validation of a One-Step Duplex RT-qPCR Assay for Analysis of RAAV Vector-Derived Gene Expression in Cynomolgus Monkeys

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Introduction: Demands for preclinical safety assessments of gene and cell therapy test articles have effectively increased the necessity for regulated vector biodistribution and vector-derived gene expression studies. Guidance documents issued from numerous international regulatory authorities recommend the use of quantitative polymerase chain reaction (qPCR) and reverse transcriptase qPCR (RT-qPCR) assays to analyze biodistribution and gene expression samples due to their highly sensitive and robust target-specific detection. Compared with qPCR, RTqPCR analysis of vector-derived gene expression is more complicated as additional parameters should be monitored. Viral vector genomic DNA contamination in RNA samples can affect overall data integrity, therefore RNA samples from the dosed animals should be tested by RT-qPCR both in the presence and absence of reverse transcriptase. RNA samples severely contaminated with vector DNA will require additional DNase I treatment(s) and reanalysis. RNA integrity should also be monitored as RNA is prone to degradation. Therefore, a selected reference gene(s) should be included in the validation. Currently, there is a complete lack of regulatory guidelines for RT-qPCR assay assessment to support vector-derived gene expression analysis. Consequently, the approach to conduct preclinical RT-qPCR method development, validation, and sample analysis can vary between bioanalytical scientists and laboratories based on their scientific interpretation. Regarding this need, we report the method development and validation of a one-step RT-qPCR assay for analysis of a recombinant AAV (rAAV) vector-derived transgene mRNA in cynomolgus monkeys and recommend parameters to evaluate during method development and validation. **Method Development, Results, and Conclusion:** Two sets of primers and probes were developed, one for rAAV genome detection and vector-derived transgene mRNA in the presence of monkey RNA, and the other for detection of endogenous reference monkey *HPRT1* mRNA. The primer and probe concentrations were optimized to meet the validation acceptance criteria. To account for inefficiencies that could occur in the mRNA to cDNA conversion process, a synthetic RNA oligo encoding the vector-derived mRNA sequence was utilized in all standard curves and quality controls (QC). Currently, vector DNA contaminated RNA undergoes a time-consuming manual repurification after additional DNase I treatment(s) which brings an associated risk of sample loss. In this study, several DNase I treatments not requiring additional purification were compared and optimized to avoid any inhibition on RT-qPCR. Our data demonstrated that TURBO DNase I removed approximately 99.9% of the spiked plasmid DNA in RNA samples while ezDNase only removed approximately 97-98% of the spiked plasmid DNA in the RNA samples. **Method Validation, Results, and Conclusion:** Five validation runs were performed with serially diluted RNA standards, negative controls, and QCs, plated in the presence of 100ng cynomolgus monkey liver RNA matrix. Each completed run met the validation acceptance criteria for target and reference gene mRNA amplification. The ULOQ, LLOQ, and LOD were 10^8, 50, and 25 single-stranded copies per reaction, respectively. Regardless of standard RNA concentration, little variation was seen in *HPRT1* Ct values among the same type of matrix RNA, showing that various amounts of target mRNA did not affect *HPRT1* gene amplification. Neither freeze-thaw cycling nor various reagent lots affected the validation run and no matrix effect was observed. The extensive evaluation of the fit-for-purpose validation parameters confirms the applicability of the RT-qPCR method for non-clinical studies of vector-derived gene expression.

1113 Gene Editing of R14del Mutation in PLN Rescues PLN-R14del-Associated Cardiomyopathy

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The Arg14del (R14del) mutation in the Phospholamban (PLN) gene is a common genetic cause of familial dilated or arrhythmogenic cardiomyopathy. PLN plays a crucial role in intracellular calcium

homeostasis of the cardiomyocyte by negatively regulating sarco/endoplasmic reticulum Ca2+-ATPase (SERCA). The R14del mutation creates a dominant active form of PLN and results in both super-inhibition of SERCA activity and PLN protein aggregation. PLN-R14del-associated cardiomyopathy has a poor prognosis, high adolescent mortality, and no effective therapy other than heart transplantation. In the last decade, CRISPR-Cas9 gene editing emerged to offer a potential new era of curing and preventing human genetic diseases. The ability to correct the specific pathogenetic driver underlying disease, the R14del mutation, through in vivo delivery of CRISPR-Cas9 components represents an exciting therapeutic frontier for addressing PLN-R14del-associated cardiomyopathy. Tenaya Therapeutics has developed a gene therapy designed to deliver both Cas9 and PLN-R14del-specific sgRNA from a single AAV vector. We first tested our gene editing therapy in patient-specific human iPSC-derived cardiomyocytes (iPSC-CMs) and found it precisely and efficiently edited the PLN-R14del allele without affecting the wild-type PLN allele. We further tested various doses of our PLN-R14del gene editing therapy in a well-characterized mouse model and found heart function of PLN-R14del mice were rescued to near wild-type levels. Our preclinical results suggest PLN-R14del gene editing may be a promising therapy for PLN-R14del-associated cardiomyopathy.

1114 Validation of a Platform Method for Quantification of Cell Lines

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Reliable quantification of viable cells is a critical aspect in the quality control of biopharmaceutical products during all drug lifecycle phases, including drug development, clinical investigation, process scale-up, GMP manufacturing, and batch release for patient use. Current challenges in the BioPharma industry include the routine execution of test methods that can provide quality data within a reasonable turnaround-time, while offering testing capabilities toward a variety of manufacturing cell lines and cell products in a setting governed by pharmaceutical regulatory requirements. As per the FDA Industry Guidance on CAR T Cell Products, it is crucial to develop and implement well-designed logistics, including the timing for sampling and testing for lot release due to the limited shelf life of these cell therapy products before product quality degrades. In addition, in-process controls such as cell viability have to be outlined in accordance with regulatory agency guidance on CMC for Human Gene Therapy Investigational New Drug Applications (IND). Considering the current biopharmaceutical needs and regulatory expectations, this study focuses on the validation of a platform method for quantifying total cell concentration, viable cell concentration, and viability on selected cell lines, using an automated cell counter. The validation strategy involves the evaluation of data with statistical parameters, which are critical to assess system suitability and demonstrate reliable testing performance according to its intended use. The ultimate goal of this work is to demonstrate a statistically based approach in the validation and establishment of an analytical method that can support time-sensitive routine testing and quantify cell lines that have biopharmaceutical relevance in the field of cell and gene therapy.

1115 Restoration of Auditory Function with Otoferlin Gene Transfer Therapy in Nonsense (Q828X) and Missense (pR1934Q and Deaf5) Models of Otof Deficiency

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Background Otoferlin is a calcium sensor protein expressed in the inner hair cells and is important for proper synaptic transmission between inner hair cells and the afferent fibers of the spiral ganglion. Biallelic loss of function mutations in the *OTOF* gene lead to congenital severe-to-profound auditory neuropathy spectrum disorder (ANSD) in both humans (DFNB9) and in mice. DFNB9 is believed to be causal in 2-3% of individuals born with hearing loss. In human patients, numerous variants of *OTOF*-related deafness (DFNB9) have been identified. We previously demonstrated that *OTOF* gene transfer therapy can restore auditory function measured by Auditory Brainstem Response (ABR) in Q828X mutant mice that leads to nonsense mutation. Here, we compared the efficacy of *OTOF* gene transfer in additional mouse models carrying missense mutations to evaluate efficacy in a wider range of *OTOF* variants. **Methods** Three different mouse models of *OTOF*-related deafness were treated with dual hybrid AAV vectors encoding *OTOF*. Otof-Q828X is a mouse model of a cognate variant (p.Q829X) characterized in Spanish and Latin American populations (Migliosi et al. 2002; Rodríguez-Ballesteros et al. 2008). The p.R1934Q mouse model was developed as a model carrying a homozygous R1939Q variant that has been characterized in Korean population (Kim et al. 2018). Lastly, Deaf5 is a mouse model harboring an ENU-induced missense mutation resulting in a single base substitution in the exon 10 of in the mouse otoferlin gene (Longo-Guess et al., 2007). For all three groups, male and female mice were administered with dual AAV vectors around 4-6 weeks of age. Restoration of auditory function was assessed at 4 weeks after administration using auditory brainstem response to tone bursts over 4-32 kHz. Transgene expression was evaluated by anti-myc tag antibody staining of cochlea wholemount preparation to distinguish from endogenous Otoferlin. **Results** In all three DFNB9 mouse models with different variants of *Otof*, the majority of mice showed restoration of auditory function measured by improvement in ABR thresholds compared to vehicle treated control mice over the entire tested range (4-32 kHz). In all groups, over 50% of animals achieved ABR thresholds within the range observed in wild type mice. In addition, transgene expression patterns will be discussed.

Conclusion These results demonstrate that *OTOF* gene transfer therapy with dual hybrid AAV can restore auditory function in various *OTOF*-deficient situations in murine models and is likely to provide benefit for DFNB9 patients in wider populations.

1116 Optimization of AAV-Mediated Gene Therapy for the Accelerated Aging Disorder-Cockayne Syndrome

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Our goal is to optimize a clinically relevant gene delivery strategy for disorders caused by pathogenic variants in the classic Cockayne syndrome genes *ERCC6* and *ERCC8*, several xeroderma pigmentosum (XP) genes *ERCC2, ERCC3, ERCC4*, and *ERCC5* that can all lead to accelerated aging and neurodegeneration - a Cockayne syndrome (CS) phenotype. This study is designed to optimize adeno-associated virus (AAV) serotype, delivery route, promoter, and dose to target specific cell types and regions of the brain that are most relevant to the treatment of CS. Using a constitutive chicken β-Actin (CBA) promoter to drive expression of a luciferase transgene fused to YFP, we compared the following AAV delivery routes: intravenous (IV) $1x10^{13}$ vg/kg body weight, intrathecal (IT) $1x10^{12}$ vg/kg body weight, and intra-cerebroventricular (ICV) $1x10^{12}$ vg/kg total dose following bilateral administrations. All studies were performed in wild-type FVB/NJ mice. For each delivery route, we compared the transduction efficiencies of two capsids: AAV9 and AAV-DJ. Luciferase expression was confirmed through *in vivo* bioluminescence imaging at regular intervals throughout the eight week study. After eight weeks, mice were euthanized and full necropsies performed. Our evaluations thus far have shown the following: (ICV) AAV-DJ demonstrated significantly increased vector genome (vg) uptake in cerebellum, spinal cord, hypothalamus, and hippocampus as compared to AAV9 and RNA transcript expression analyses correlated well. (IT) AAV-DJ demonstrated significantly increased vg uptake in the cortex as compared to AAV9 and RNA transcript expression analyses correlated well. (IV) AAV-DJ demonstrated significantly increased vg uptake in cerebellum, spinal cord, hypothalamus, and hippocampus as compared to AAV9 and RNA transcript expression analyses is underway. Evaluations of other tissues and biodistribution assessments of protein expression within CNS cell types are ongoing. As an additional layer of control over expression can be conferred through promoter choice, we are evaluating a panel of existing promoters and novel mini-promoters to evaluate their relative efficacy. Information gained from these collective studies will guide the design of therapeutic vectors for CS.

1117 Development of Liver De-Targeted AAVrh74 Vectors with Increased Transduction Efficiency in Mouse Muscles Following Systemic Administration

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Solid Biosciences reported serious adverse events such as complement activation and thrombocytopenia causing renal damage and cardiopulmonary insufficiency in a gene therapy rial of Duchenne muscular dystrophy using AAV9 vectors. Pfizer, also using AAV9 vectors, reported several serious adverse events such as acute kidney injury involving atypical hemolytic uremic syndrome and thrombocytopenia, and the death of a patient. Sarepta Therapeutics reported vomitting as the only adverse event in 50% of patients using AAVrh74 vectors, indicating that these vectors are safer. However, in pre-clinical studies with mdx mice (*Hum. Gene Ther.*, 32: 375-389, 2021), greater than a log higher genome copy numbers of AAVrh74 vectors were sequestered in mouse liver than any other muscle tissues (gastrocnemius, tibialis anterior, triceps, quadriceps, and diaphragm), thereby limiting their availability for muscle transduction. We also observed abundant transduction of mouse liver with the AAVrh74 vectors injected intravenously in C57BL6/J mice at $1x10^{10}$ and $1x10^{11}$ vgs/mouse (in two different experiments), 2-week post-vector administration (**Figure 1A**). Thus, it is of paramount importance to develop strategies to de-target AAVrh74 vectors from the liver. In the present studies, we generated liver de-targeted AAVrh74 vectors based on a published study (*Sci Transl Med*, 12: 2020), in which liver-tropic AAV serotype vectors such as AAV2, AAV3, and AAV8, which contain a threonine (T) residue at position 265 within the variable region I (VR-I) of the capsid, were documented to play a key role in liver transduction. Since the T265 residue is also conserved in AAVrh74 (**Figure 1B**), using site-directed mutagenesis, we generated a T265-deletion mutant of AAVrh74 vector. WT- and T265del-AAVrh74-FLuc vectors were injected intravenously at $1X10^{12}$ vgs/mouse (n=5), and whole-body bioluminescence imaging was performed 2-weeks post-vector administration. As can be seen in **Figure 1C**, the T265del-AAVrh74 vectors were significantly de-targeted from the liver ($p<0.05$). More interestingly, the transduction efficiency of the T265del-AAVrh74 vectors was significantly increased in the muscle ($p<0.05$) (**Figure 1D**). The use of liver de-targeted capsid- and genome-modified optimized (Opt) AAVrh74 vectors may prove to be safe and effective in the potential gene therapy of human muscular dystrophies at reduced doses.

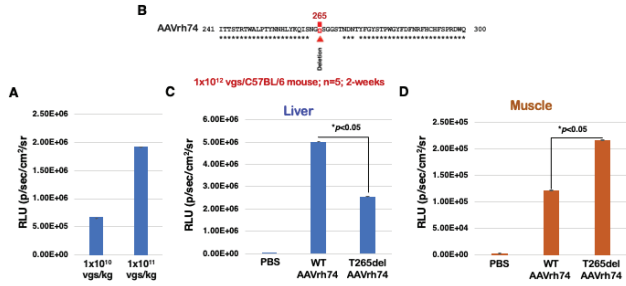

Figure 1: (A) Robust liver transduction with AAVrh74-CBA-FLuc vectors in C57BL/6 mice. **(B)** T265 is conserved in AAVrh74, and its deletion leads to significant liver de-targeting following intravenous delivery **(C)**, and a corresponding increased transduction of the mouse gastrocnemius muscle **(D)**.

1118 Preclinical Development of LYL119, a ROR1-Targeted CAR T-Cell Product Candidate Incorporating Four Novel T-Cell Reprogramming Technologies to Overcome Barriers to Effective Cell Therapy for Solid Tumors

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For solid tumor cell therapy to be effective, new strategies are needed to improve T-cell activation, persistence, and durable function. We have developed four stackable reprogramming technologies to address these issues: 1) genetic overexpression of the activator protein 1 (AP-1) family transcription factor c-Jun to reduce chimeric antigen receptor (CAR) T-cell exhaustion and improve functional activity; 2) nuclear receptor subfamily 4A (NR4A) gene knock-out (KO) because NR4A transcription factors may contribute to exhaustion and reduce T-cell function by limiting expression of AP-1-regulated genes; 3) Epi-R™ manufacturing protocols to promote stem-like characteristics; and 4) Stim-R™ technology, a synthetic cell mimic that presents anti-CD3 and anti-CD28 signaling molecules to optimize T-cell activation during manufacturing. The resulting ROR1-targeted CAR-T cell product, LYL119, combines these T-cell reprogramming technologies to create potent CAR T cells with durable function (Figure 1A). Healthy donor T cells were manufactured with the Epi-R protocol, activated with a conventional T-cell activation reagent or Stim-R technology, and transduced with a tri-cistronic lentiviral vector encoding a ROR1 CAR, c-Jun, and truncated EGFR. The *NR4A3* gene was disrupted using CRISPR/Cas9 ribonucleoprotein delivery via electroporation. We first evaluated combining NR4A3 KO and c-Jun overexpression in the Epi-R protocol in ROR1 CAR T cells. NR4A3 KO + c-Jun ROR1 CAR T cells consistently demonstrated significant additive benefit with superior cytotoxic activity, prolonged cytokine production, and reduced surface expression of inhibitory receptors after repetitive *in vitro* antigen stimulation, suggesting a mechanism of resistance to exhaustion-induced dysfunction. In the H1975 *in vivo* model, NR4A3 KO + c-Jun CAR T cells demonstrated robust anti-tumor efficacy with activity at a 7-fold reduced CAR T-cell dose (lowest dose, p<0.005, 3 donors) and more than 20-fold greater CAR T-cell expansion in blood compared to control + c-Jun CAR T cells. Even in the difficult-to-treat A549 *in vivo* model, NRA43 KO + c-Jun CAR T cells demonstrated improved tumor control compared to control (p<0.0001). Importantly, NR4A3 KO and c-Jun overexpression did not induce uncontrolled CAR T-cell expansion *in vitro* or *in vivo*. We then evaluated combining Stim-R and Epi-R technologies for ROR1 CAR T production. These cells showed prolonged cytotoxicity and cytokine production *in vitro*, and significantly improved tumor control (p<0.0001), CAR T cell expansion in blood (31-fold, p<0.0001), and overall survival (p<0.0001) in the H1975 *in vivo* model compared to control CAR T cells. Finally, we incorporated NR4A3 KO + c-Jun and Stim-R into the Epi-R protocol and showed that combining our four reprogramming technologies in ROR1 CAR T cells showed additive benefits to prolong *in vitro* cytotoxicity using A549 tumor cells compared to controls (Figure 1B). Together these data suggest that stacking these four technologies can limit exhaustion and has the potential to provide effective and durable ROR1 CAR T-cell functional activity in patients with ROR1$^+$ solid tumor malignancies.

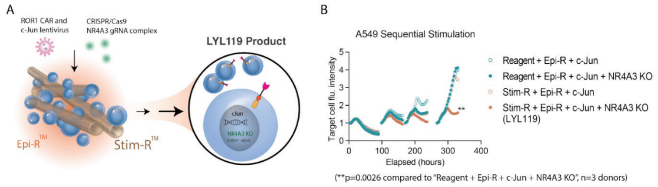

Figure 1. (A) Schematic of LYL119. (B) LYL119 demonstrates prolonged cytotoxicity in vitro (1 of 3 donors shown).

1119 *In Situ* CAR Therapy Using oRNA Lipid Nanoparticles Regresses Tumors *In Vivo*

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LNP-mediated delivery of long coding RNA has been clinically validated for vaccines and gene editing. We have been developing a novel, synthetic, circular coding RNA platform (oRNA technology) which exhibits significant improvements in production, expression and formulation compared to mRNAs. Lacking the cap structure of mRNA, our oRNA technology uses a proprietary sequence-based IRES element to initiate protein translation in target cells. At the same time, *ex vivo* generated chimeric antigen receptor (CAR) T cell therapies have had tremendous success in treating hematologic malignancies, yet manufacturing, safety and efficacy challenges remain. We have been combining oRNA technology with novel immunotropic LNPs to address these challenges by creating off-the-shelf yet "autologous" *in situ* CAR (isCAR™) therapies. Our immunotropic LNPs show preferential biodistribution to the spleen with oRNA reporter expression detected in multiple immune

cell subsets including T cells, macrophages and NK cells. Delivery to immune cells is preserved across mice, rats and non-human primates. *In vitro*, expanding human T cells expressing an anti-human CD19 CAR oRNA show potent and sustained cytotoxicity and pro-inflammatory cytokine production compared to controls. To maximize protein expression, we developed the FoRCE (Formulated oRNA Cell-based Evaluation) platform: a robust high-throughput platform that enables parallel arrayed synthesis, purification, lipid nanoparticle (LNP) formulation, and cell-based screening of oRNAs. We applied FoRCE to ~3,000 unique oRNAs containing UTRs extracted from viral genomes and discovered hundreds of IRESs that drive translation from synthetic oRNA in primary human cells across diverse tissues. Select IRESs from this screen drove high levels of CAR expression and cytotoxicity in primary human T cells that were significantly elevated compared to modified mRNA and earlier IRESs. Optimizing the IRES and coding sequences translated into a 20-fold increase in efficacy in mice treated with the corresponding LNP-oRNAs in a human PBMC-engrafted NALM6 tumor-bearing mouse model. The optimized LNP-oCAR enabled weekly dosing at clinically relevant dose levels producing well-tolerated, robust and reproducible efficacy across multiple PBMC donors. Tumor regression was dose-dependent. oRNA-enabled isCAR therapies promise a transient, re-dosable and scalable immune cell therapy without requiring immunodepletion for the treatment of cancer.

1120 Lack of the Classical Complement Pathway in C1q-Deficient Mice Results in Reduced Humoral Responses but Enhanced Cytokine Induction after AAV Dosing

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High doses of systemically delivered AAV have been associated with serious adverse events like thrombotic microangiopathy (TMA) and atypical hemolytic uremic syndrome (aHUS) in clinical trials. Both syndromes are associated with excessive activation of the complement system. Subsequent studies demonstrated that AAV-induced complement activation is antibody-dependent, implicating the classical pathway (Smith et al., 2022). Because complement is one of the first elements of immunity to recognize AAV, we sought to understand better how activation of classical complement and generation of its split products enhances downstream immune responses. To improve our understanding of how classical complement influences immune responses to high-dose AAV, we dosed C1qa knockout mice (Jackson Labs, *B6(Cg)-C1qatm1d*(EUCOMM)Wtsi/TennJ). C1q is the first complement component to recognize antibodies on opsonized antigens and, therefore the first complement protein to recognize AAV-antibody immune complexes. We dosed C1q homozygous knockout mice and *C57Bl6/J* wildtype mice with AAV9-CMV-GFP (1.2E14vg/kg) with an initial dose to prime adaptive immunity followed by a second dose of AAV9-CMV-mCherry four weeks later. Blood was collected before dosing and then 5 hours and 2 weeks after each dose. The humoral response to AAV dosing was tested on high-content protein microarray chips and showed that while both WT and *C1q-/-* mice induced anti-capsid IgM responses, only WT mice had a linear increase in anti-AAV-IgG while the induction of anti-AAV IgG was blunted in *C1q -/-* mice. These data suggest that *C1q-/-* mice show a defect in IgM to IgG class switching. Previously, only the complement split product C3d was associated with the process of switching IgM to IgG classes, which cannot be directly induced by classical complement activation. In spite of the blunted humoral response *C1q-/-* mice showed a much stronger induction of monocyte-derived chemokines than WT mice. Chemokines such as CXCL10 and MCP1 were induced five hours after the second AAV dose in both genotypes of mice. This enhanced chemokine induction after the second administration of AAV was observed in cohorts of mice dosed at both four-week and two-week intervals. The aim of our future research is to understand the mechanisms by which classical complement influences switching between immunoglobulin classes and why induction of monocyte-derived chemokines are enhanced in the absence of C1q.

1121 TARGET-AAV: Tissue-Specific Alternative Splicing to Regulate Globally Expressed Therapeutics

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Systemic delivery of AAV to replace defective genes has proven to be an effective therapeutic approach. However, the large vector doses required for therapeutic efficacy can place extreme burdens on off-target tissues such as the liver. In some cases, the undesired transduction and subsequent transgene expression in these tissues can have devastating toxic effects in lieu of the desired therapeutic outcome. Improvements in capsid tropism and promoter sequences have alleviated some off-target concerns, but transgene expression is still largely un-regulated. Further, tissue-specific cargo regulation may be required to prevent toxicity in diseases where dissimilar gene expression levels are necessary for therapeutic efficacy, such as muscular dystrophies where heart and skeletal muscle are often both affected. Gene expression regulation by RNA splicing has been well studied over many decades. RNA splicing provides an added regulatory element that is tunable and well-regulated. Here, we describe the exploitation and application of RNA splicing mechanisms to regulate gene therapy expression in a tissue-specific manner. Others have demonstrated the possibility of RNA-mediated expression control in different cell types of the brain or through the use of small molecule splicing modulation. In a parallel approach, our lab has developed a high throughput alternative exon identification pipeline coupled with *in vivo* screening and selection of alternative exon libraries to identify transgene expression with tissue-specific expression patterns and behaviors. Using **t**issue-specific **a**lternative splicing to **r**egulate **g**lobally **e**xpressed **t**herapeutic AAV (TARGET-AAV), we have identified alternative exons that, when expressed in combination with AAV transgene delivery, can enhance tissue-specific expression levels and patterns.

1122 Therapeutic Gene Editing of CFTR to Treat Cystic Fibrosis, Using Nanoparticle Formulations for the Delivery of CRISPR/Cas9

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Background:Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The dysfunctional CFTR protein leads to thickened mucus, bacterial infections, and reduced lung function. Modulators are effective treatments for many CFTR genotypes, but ~20% of patients remain untreatable. Aims:The aim was to develop a nanoparticle formulation to deliver CRISPR/Cas9 to therapeutically edit *CFTR* mutations *in vitro*, and to investigate the effect of cellular obstacles to transfection including endocytic uptake, endosomal escape and nuclear entry on transfection efficiency. As CF involves inflammation of the lung, a further aim was to determine whether delivery exacerbates inflammation. Methods:Plasmid DNA (pDNA) was packaged into receptor-targeted nanocomplexes (RTNs), comprising liposome and a targeting peptide. 16HBE14o- (16HBE) and BMI-1 transduced CFBE F508del cells were transfected with RTNs, and IL-6 protein secretion measured by ELISA as a marker of inflammation. Nuclear uptake and gene expression were evaluated by confocal microscopy, flow cytometry and luciferase assays. Endosomal escape was assessed by adding chloroquine to the transfection. Results:The optimal RTN formulation for transfecting both cell types comprised a liposomal formulation of ditetradecyl trimethyl ammonium propane (DTDTMA), dioleoyl L-α phosphatidyl ethanolamine (DOPE) at a 1:1 molar ratio, combined with a peptide, K_{16}-GA-CSERSMNFCG. Transfection efficiency with GFP pDNA was 21.7% in 16HBE cells and 37.1% in CFBE cells, and the uptake efficiency of Cy5-labelled pDNA was 62.4% and 99.1% respectively. Chloroquine, an enhancer of endosomal escape, reduced RTN transfection efficiency in 16HBE cells. The microscopy showed that many RTNs were perinuclear 48 hours post-transfection in 16HBE cells. mRNA transfection efficiency was significantly higher than pDNA in CFBE cells but not in 16HBE cells. RTN transfection did not significantly increase IL-6 levels, a marker of inflammation, but dexamethasone reduced IL-6 levels in 16HBE cells. Conclusion:Taken together, this work suggests that the major obstacles to transfection were cellular and nuclear uptake in 16HBE cells, and nuclear import, transcription, nuclear export and/or translation in CFBE cells. There was no evidence of an inflammatory response to RTN transfection. Further steps must be taken to develop this gene editing approach before translation *in vivo*.

1123 An Erythroid-Specific Lentiviral Vector Improves Anemia and Iron Metabolism in a New Model of Alas2 Knock-Out X-Linked Sideroblastic Anemia (XLSA)

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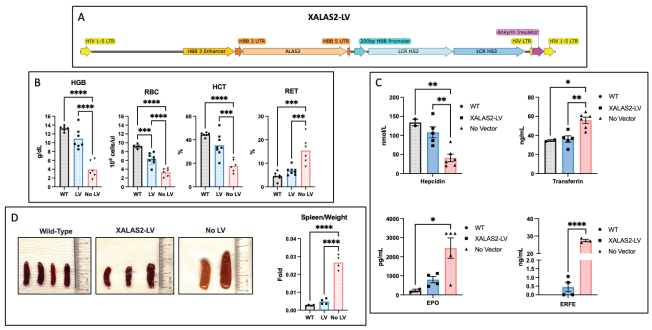
X-linked sideroblastic anemia (XLSA) is the most common form of congenital sideroblastic anemia caused by a germline mutation in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene. Patients with XLSA are predominantly hemizygous males who exhibit hypochromic microcytic anemia accompanied by systemic iron overload. *Ex-vivo* hematopoietic stem cell (HSC) gene therapy using a lentiviral vector (LV) represents a potential therapeutic option, improving patients' conditions by supplying a functional copy of the ALAS2 cDNA. To control the expression of ALAS2 cDNA in an erythroid fashion, we generated a lentiviral vector expressing human ALAS2 under the control of the human β-globin promoter and elements of the locus control region (LCR). We also included an insulator in the 3' self-inactivating long terminal repeat (SIN-LTR) to enhance safety (Fig.1A). Constitutive knock-out mice of ALAS2 are not viable at birth. Hence, we generated a conditional KO flanking a critical region of the *Alas2* mouse gene with LoxP sites. Untransduced or transduced R26-CreER^{T2}-ALAS2$^{fl/Y}$ Lin- selected cells were then transplanted into lethally irradiated recipients. Eight weeks post engraftment, the *Alas2* gene was deleted through the expression of Cre, initiated by the administration of tamoxifen. Mice treated with the LV and controls (*Alas2*-KO animals) showed significant CBC differences. While the hemoglobin and RBC levels were improved or corrected in the LV-treated group, the non-treated group showed fatal anemia (Fig.1B), with low RBC levels and hematocrit (HCT). Accordingly, the untreated group has increased erythropoietin levels and reticulocyte counts because of ineffective erythropoiesis and severe anemia (Fig.1B-1C). The ineffective erythropoiesis was characterized by extra-medullary erythropoiesis and severe splenomegaly, while XALAS2-LV almost completely normalized these parameters in treated mice (Fig.1D).In non-treated animals, the resulting anemia was also accompanied by increased ERFE, the erythroid regulator of hepcidin, and decreased HAMP levels. As expected, low HAMP levels were associated with increased serum iron and transferrin saturation levels, in line with increased iron absorption, iron overload (Fig. 1C), and ineffective erythropoiesis. These parameters were improved or normalized in the XALAS2-LV-treated group.These data show that the fatal anemia in *Alas2* KO animals can be improved or corrected by XALAS2-LV. Notably, animals rescued by XALAS2-LV showed vector copy numbers (VCN) in the range of 0.5-1.4, indicating that a small number of integrations were sufficient to rescue *Alas2*-KO animals. As XLSA patients still exhibit hypomorphic or residual expression of the ALAS2 gene (as the complete absence is incompatible with survival), we hypothesize that minimal gene transfer or mini-transplants with HSCs treated with XALAS2-LV could be curative in this disease.



A. XALAS2 Lentiviral Vector, developed with the use of β-globin promoter, regulatory and safety elements; B. CBC results showing severe and fatal anemia characterized by very low RBC synthesis and low HCT levels; C. Non-treated group shows HAMP low levels, due to an increase of ERFE levels confirming increased transferrin saturation, iron overload, and ineffective erythropoiesis; D. XALAS2-LV normalizes severe splenomegaly and extramedullary erythropoiesis. A control group (animals engrafted with R26-CreER^{T2}-HSCs) was also included in this study.

1124 An Evolved AAV Variant Enables Efficient Genetic Engineering of Murine T Cells and the Modeling of *Trac*-CAR-T Cells in Immunocompetent Tumor Models

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Precise targeting of large transgenes to T cells using homology-directed repair (HDR) has been transformative for adoptive cell therapies and T cell biology. Delivery of HDR templates via adeno-associated virus (AAV) has greatly improved knockin efficiencies, but the tropism of current AAV serotypes largely restricts their use to human T cells employed in immunodeficient xenograft mouse models. To enable targeted knockins in murine T cells, we evolved Ark313, a synthetic AAV that exhibits very high transduction efficiency in murine T cells. To identify the essential host factors for Ark313 transduction, we performed a genome-wide knockout screen and identified the MHC-I molecule QA2 as an essential factor for Ark313 infection. We demonstrate that Ark313 can be used for nucleofection-free DNA delivery, CRISPR-Cas9-mediated knockouts, and targeted integration of large transgenes with up to 75% knockin efficiency. Ark313 enables preclinical modeling of *Trac*-targeted CAR-T and transgenic TCR-T cells in immunocompetent models. We compared murine *Trac*-CAR-T cells and gRV CAR-T cells in a solid lung tumor model and *Trac*-targeted CAR T cells displayed increased tumor infiltration and significant improvement in survival compared to gRV CAR-T cells. Next, we assessed Ark313 for in vivo engineering of T cells. We first injected a single dose of Ark313 expressing Cre recombinase in Ai9 fluorescent reporter mice and achieved permanent genetic changes in >20% of splenocyte T cells in vivo. Additionally, Ark313 tropism for T cells was associated significant liver de-targeting relative to parental AAV6. We then injected Ark313 expressing a *Trac*-targeting sgRNA in Cas9 expressing mice and achieved TCR KO in >10% T cells in vivo. These results demonstrate the ability to perform CRISPR based gene editing in T cells in vivo using AAV delivery. These new advancements open avenues for functional genomics in vivo and experimental modeling of gene edited T cells in immunocompetent settings.

1125 Increasing Erythropoietic Output from Genome-Edited Hematopoietic Stem and Progenitor Cells Using a Truncated EPO Receptor

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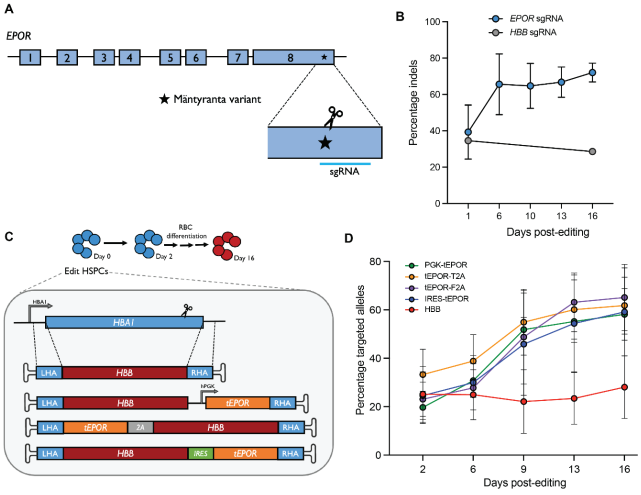
Genome-edited hematopoietic stem and progenitor cells (HSPCs) yield genome-corrected cells of all lineages, yet the only cell type of clinical relevance to the hemoglobinopathies is the red blood cell (RBC). With this in mind, we aimed to develop a novel genome editing strategy to enrich edited HSPCs within the erythroid lineage. This could allow low HSPC chimerism in the bone marrow to yield high levels of RBC chimerism in the bloodstream, thereby amplifying the efficacy of hemoglobinopathy treatments in the clinic. From human clinical genetics we know truncations in the erythropoietin receptor (EPOR) cause benign erythrocytosis, yielding non-pathogenic hyper-production of RBCs. This variant was first identified in a cohort that included a Finnish Olympic cross-country skier and does not cause any clinical pathology besides an elevated hemoglobin. We hypothesized we could recapitulate this natural variant using CRISPR/Cas9 genome editing to create insertions/deletions (indels) at the *EPOR* locus near the site of the Olympic skier's mutation (Mäntyranta variant) (Figure 1A). We demonstrated increased erythropoietic output from cells containing truncating mutations in *EPOR*, shown by an increase in indel formation throughout erythroid differentiation using an *EPOR* single guide RNA (sgRNA) versus no increase in a control sgRNA targeting *HBB* (Figure 1B). Because not all indels created caused truncations in *EPOR*, we next explored whether we could amplify this proliferative effect by inserting a truncated EPOR (*tEPOR*) cDNA in the endogenous locus and found this strategy also enriched for cells with the truncation as they developed during erythroid differentiation. Next, we investigated whether the *tEPOR* cDNA could be targeted to the *CCR5* safe harbor locus under ubiquitous expression or to the *HBA1* RBC-specific safe harbor site imparting high levels of erythroid specific expression. We demonstrated that integration of the *tEPOR* cDNA at both loci produced strong enrichment of edited alleles in an EPO-dependent manner when put through erythroid differentiation. Lastly, we sought to pair the *tEPOR* cDNA with a clinical edit for β-thalassemia to boost production of disease-corrected RBCs. We showed that through use of bicistronic cassettes (Figure 1C) we were able to drive a >2-fold enrichment of edited alleles over the course of differentiation (Figure 1D). Additionally, we were able to pair the *tEPOR* cDNA and clinical edit for β-thalassemia through multiplexed gene editing at *EPOR* and *HBA1*, respectively, and again demonstrated enrichment of edited alleles. Ultimately, we believe this work has the potential to amplify the efficacy of gene and cell therapies for blood disorders currently in the

clinic, including both allogeneic and autologous HSPC transplantation. Further, as low chimerism in the bone marrow could produce high levels of peripheral RBCs using our strategy, it has the potential to reduce or eliminate the high-morbidity myeloablation regimens currently required for functional cure of blood disorders. This work, in effect, has the potential to improve the safety and accessibility to currently available treatments for the millions of patients affected by the hemoglobinopathies worldwide.

Figure 1



1126 3D Printed Hydrogel Scaffolds Generated Using Continuous Liquid Interface Production for the Delivery of Neural Stem Cells to Treat Glioblastoma

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Background: Neural stem cell (NSC) therapy is a promising new platform for treating glioblastoma (GBM), an aggressive and deadly malignant brain tumor associated with a median survival of only 12-15 months. However, NSCs implanted directly into the GBM resection cavity in mice are cleared from the implant site within 3-5 days, leading to insignificant improvements in overall efficacy. We hypothesized that improving NSC persistence via biomaterial encapsulation in the GBM cavity would lead to better therapeutic efficacy; however, NSC migration from the material must be maintained. To satisfy these conditions, we utilized continuous liquid interface production (CLIP) to 3D-print biocompatible hydrogel scaffolds, on which NSCs would be seeded. We hypothesize that the scaffold architecture could act as a physical barrier to NSC clearance, permitting enhanced *in vivo* NSC viability without compromising cell migration. Methods: CLIP was used to generate a 3D gelatin methacryloyl (GelMA)- and poly(ethylene glycol) diacrylate (PEGDA) hydrogel scaffold, cylindrical in shape with a latticed design (Fig. 1). NSCs were seeded onto the external scaffold surfaces via a combined static and centrifugal seeding method. NSC viability on the scaffold was quantified with BLI imaging, and SEM imaging was used to evaluate cell confluency, attachment, and morphology of the NSCs on the scaffold over time. Fluorescence imaging was used to assess cell migration patterns of NSCs to GBM tumors from CLIP scaffolds. *In vivo* persistence was studied by implanting NSC-loaded CLIP scaffolds into mock resection cavities in athymic nude mice, after which NSCs were tracked via BLI. Results: NSCs seeded onto the biocompatible CLIP scaffolds, following 14 days in culture the NSCs proliferated to a density over 30-fold higher than the initial seeding density. SEM images depicted changes to NSC morphology on the scaffolds over time, in which the cells initially took on a spherical shape that changed to a flattened morphology as they attached firmly to the scaffold material. In vitro migrations studies indicate that NSCs cultured on CLIP scaffolds could leave the scaffold and migrate to GBM cells. *In vivo* persistence studies showed that NSCs on CLIP scaffolds implanted in the brains of healthy mice, continued to proliferate to 20% higher than the seeded dose by day 6, then exhibited a slow decline in persistence until day 15. Contrastingly, NSCs injected into the resection cavity in PBS showed an immediate and sustained decrease in persistence, and by day 6 over 70% of the initial NSC signal was lost. Future Directions: In the future, we will evaluate the ability for NSCs on CLIP scaffolds to produce a more robust therapeutic effect in our model of GBM resection in mice.

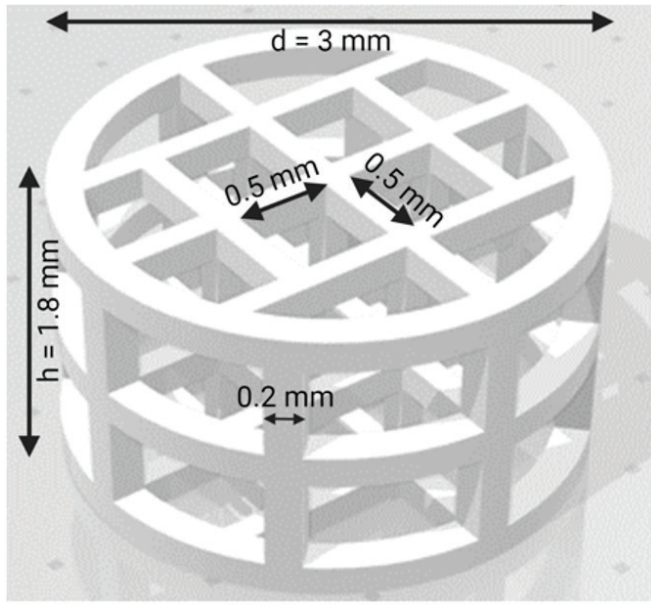

Figure 1. Rendering of CLIP scaffold design.

1127 Measuring Small Molecule Improvements in Genome Editing for Pyruvate Kinase Deficiency Using DNA Barcoding Templates

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Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in PKLR gene. PKD-erythrocytes suffer from energy imbalanced caused by reduction of erythroid pyruvate kinase (RPK) activity. PKD is associated with hemolysis, reticulocytosis, splenomegaly and iron overload, and may be life-threatening. We developed a knock-in gene editing strategy by combining ribonucleoprotein electroporation and adeno-associated viral vector (rAAV6) donor delivery, getting relevant editing in hematopoietic stem and progenitor cells (HSPCs) from mobilized peripheral blood (mPB). Since HDR gene editing is a homogeneous modification of the genome, clonal and cell tracking studies of edited cells cannot be performed as it can with lentiviral marking. Thus, we developed a donor DNA barcode AAV6 library to allow the clonal dynamics of genome edited cells to be determined and used the assay to assess potential improvements in genome editing efficacy. Here we describe how additional modifications in gene editing protocol increase the targeted integration frequency to~70%. First, changes in the cell concentration in the procedure increase the editing 2-fold. Second, we tested nine different compounds. Two of them, one involved in the inhibition of DNA-PK pathway which can act as an inhibitor of NHEJ pathway and a specific inhibitor of HDAC class I/II, render good improvements. With the use of the DNA-PK pathway inhibitor, we increased 1.5-fold PKLR gene editing in mPB cells. There were no differences in the number of individual clones *in vitro*. When the inhibitor of the HDAC class I/II was used, we did not observe any increase in targeted integration, but when we combined both, targeted integration increases 1.8-fold with respect to untreated cells. The combination of both compounds maintains the numbers of individual clones *in vitro.* There were no changes in viability, *in vitro* expansion capabilities or total colony forming ability of edited $CD34^+$ cells in any of the combinations. We are currently assessing the number of targeted integrated HSPC clones engrafted in immunodeficient mice to evaluate their effect in the most primitive HSPC compartment. To sum up, we were able to enhance *PKLR* gene targeted integration and maintain the clonal repertoire *in vitro* by adding these enhancers. These data would facilitate the clinical use of gene targeted integration therapy for PKD.

1128 Targeting the Hepatitis B cccDNA with the hfCas12Max Nuclease to Eliminate Hepatitis B Virus *In Vitro* and *In Vivo*

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Background: Hepatitis B virus (HBV) is a hepatotropic, non-cytopathic DNA virus that generates a covalently-closed-circular-DNA (cccDNA) intermediate in the nucleus of infected cells as well as integrated sequences that act as transcription templates for viral proteins. Antiviral therapies with nucleoside analogues inhibit replication of HBV DNA in capsids present in the cytoplasm of infected cells, but do not reduce or destroy nuclear cccDNA. Here, we describe a potential curative approach using a proprietary highly specific engineered Cas12i nuclease (hfCas12Max) targeting the hepatitis B virus (HBV) genome. **Methods**: Effective guide RNAs (gRNAs) were firstly selected and screened in PiggyBac (PB)-HBV-HEK293 system. In addition, functional gRNAs screen was performed in HBV-integrated cell lines and HBV-infected primary human hepatocytes (PHHs). To evaluate the hfCas12Max nuclease *in vivo*, we developed episomal adeno-associated virus (AAV) mouse model containing 1.3-fold HBV genome serving as a surrogate for cccDNA. Clinically relevant delivery was achieved through systemic administration of lipid nanoparticles (LNP) containing hfCas12Max mRNA and gRNAs. **Results**: Lead gRNAs targeting hepatitis B surface antigen (HBsAg) and other related genes were identified in HBV-integrated hepatic cell lines. Through the LNP delivery, hfCas12Max targeting resulted in a significant reduction of cccDNA, HBV-DNA, and HBsAg in HBV-infected PHHs (Fig. 1A-C). In the AAV-HBV mouse model, we observed sustained reductions in HBsAg expression (~2log) and serum HBV DNA (~3log) after LNP-mediated systemic delivery of the CRISPR- hfCas12Max system (Fig. 2A-B). **Conclusions**: Our data suggest that the CRISPR- hfCas12Max system could disrupt the HBV expressing templates both *in vitro* and *in vivo*, indicating its potential in eradicating cccDNA toward a potential cure for HBV.

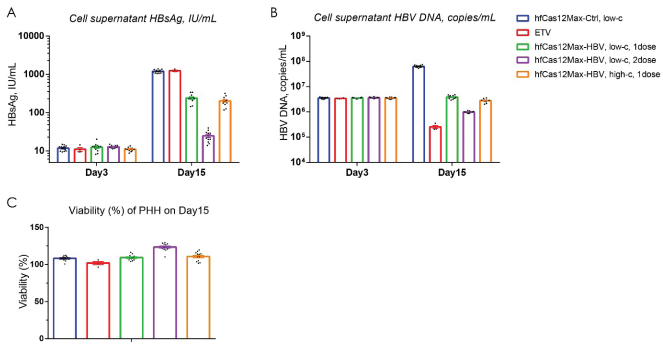

Figure 1. LNP-mediated delivery of hfCas12Max mRNA and gRNAs reduces HBV viral parameters in primary human hepatocytes (PHH). A, B) HBV replication assessed by HBsAg ELISA and HBV DNA qPCR in PHH supernatant. Cas12i-targeting leads to the efficient reduction of HBsAg (A) and HBV DNA (B). C) The viability of PHH cells in each group was unaffected on day 15.

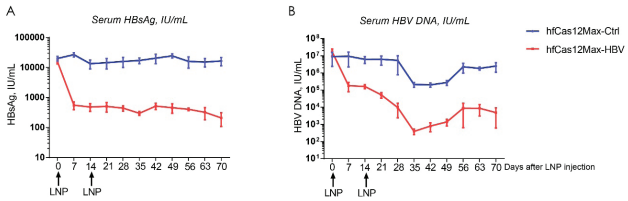

Figure 2. LNP-mediated delivery of hfCas12Max mRNA and gRNAs leads to sustained reduction of viral markers in AAV-HBV mouse model. A, B) The AAV-HBV mouse model was used in the *in vivo* pilot study. 4 weeks after hydrodynamic injection with AAV8-1.3-fold HBV genome, mice received two doses (2x) of the hfCas12Max-targeting reagents (mRNA & gRNA formulated into LNP). hfCas12Max nuclease treatment resulted in sustained reductions in HBsAg expression (~2log) (A) and HBV DNA (~3log) (B) in serum.

1129 Quality Control Pipeline for AAV Production

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Recombinant Adeno Associated Vectors (AAV) are used to deliver therapeutic genes to target cells throughout the body for gene therapy applications. To ensure consistency and purity of each batch of vector, quality control in AAV packaging and purification is crucial. Several factors can affect the therapeutic efficacy of AAV vectors due to a lack of quality control. Differences in quality can be due to process and product-related contamination, packaging efficiency and rearrangements or truncations of the packaged DNA. Our lab is developing a pipeline to establish quality control for all AAV preparations made in-house or obtained from companies, to improve the consistency and reliability of our AAV-based studies. Controls are included to enable the assessment of empty/full ratios of AAV preps. Standards purchased from Virovek allowed us to generate a standard curve of the empty/full ratios that is compared to our samples. Our evaluation of an unknown sample begins by assessing diluted crude lysates after packaging using qPCR to determine the integrity of the packaged product and to give an estimate of the titer before purification. After purification, the final genome titer is determined by ddPCR. The protein titer is determined by BCA and ELISA to quantify the concentration of capsid proteins then evaluated by Coomassie-stained polyacrylamide gels to confirm the protein purity and VP ratio of the sample. The packaged DNA is evaluated by alkaline gels to confirm the size of the packaged material and to provide a crude estimate of purity. If there are unusual bands then we perform long read sequencing and/or perform qPCR for the most common DNA contaminants. Capillary electrophoresis using the Jess system (ProteinSimple, San Jose, CA) is used to assess both VP proteins and dsDNA in tandem. The westerns are performed using a primary antibody specific to a common region in VP1/VP2/VP3 and another antibody to dsDNA. Good detection of both transgene and VP protein are achieved using minute amounts of our AAV samples. For each assay, we load based on total protein concentration. We have been troubleshooting the optimal approach to preparing AAV preps for each analysis of both the capsid and transgene for analysis. Analysis requires either a detergent or boiling to separate the AAV capsid proteins and to release the genome. Ultimately, we aim to identify the optimal approach to analyze the empty/full ratio and purity for each AAV preparation to determine which assays predict the efficacy of AAV for neuromuscular applications.

1130 Emerging Maxims for the Ethics of Preventive Human Gene Editing

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Introduction: Over the past decade, the field of gene editing research has exploded thanks primarily to CRISPR technologies. As the biomedical implications of this research have become apparent, recent years have seen a global wave of efforts to develop anticipatory guidance for various sorts of human gene editing applications. But for the most part, they do not address research aimed at somatic cell human gene editing for the purpose of prevention, or strengthening human resilience in the face of genetic, environmental, and infectious disease threats. Since, in principle, this prospect triggers human enhancement concerns, those involved in this research have been challenged to come up with distinctive safeguards and criteria, "moral maxims," to justify their efforts. In this presentation, we report on three of these maxims and the questions they raise for further bioethical analysis and public discussion. Methods: We identified these maxims in two ways. First, we conducted interviews with 92 gene editing scientists and policy makers about their views on different uses of gene editing and potential ways gene editing research should be regulated. Interviews were recorded and transcribed verbatim. Second, we analyzed the ongoing global policy discussions on human gene editing research regarding preventive interventions that raise enhancement concerns, including reports from the World Health Organization, US National Academies of Sciences, Engineering and Medicine, and the European Commission. Results: We identified three sets of governance prescriptions in the efforts by scientists, science policy makers, and bioethics scholars to address the challenge that preventive uses of gene editing pose for the traditional wariness of genetic enhancement: the "privileging prevalence" maxim; the "privileging transience" maxim, and the "privileging public engagement" maxim. The privileging prevalence maxim advocates that only common functional variants (vs. rare variants, variants from non-human animals, and artificial variants) should be used to improve disease resistance, even if the alternatives could be more effective in achieving preventive goals. The privileging transience maxim encourages that any preventive human gene editing interventions should be either reversible or temporary. Finally, the privileging public engagement maxim prescribes that any policy decisions about the interface between prevention and enhancement should depend on active public participation and "broad societal consensus." Conclusions: We will conclude the presentation with a discussion of the ethical considerations and questions raised by these maxims. For instance, given the diversity of human variation that we otherwise seek to respect and protect, why should the most prevalent traits be privileged? What rules should govern the "dialing down" of preventive enhancements in application settings? And how do logistics, jurisdiction, and authority influence public engagement? If these maxims are used, they have the potential to influence policy and practice, and therefore deserve careful scrutiny.

1131 ABO-503, a Novel Gene Therapy for Treatment of X-Linked Retinoschisis

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X-linked retinoschisis (XLRS) is a rare, monogenic disease that results in severe visual impairment. The disease is caused by mutations in the *RS1* gene, which is normally secreted by retinal photoreceptors and bipolar neurons and functions to mediate cell-cell adhesion. Affected individuals develop cavities between the layers of the retina where these interactions are disrupted, leading to discontinuity in the retinal circuitry and the irreversible loss of photoreceptor cells. The current standard of care for XLRS patients is limited to palliative measures, but because the genetics of the disease are well understood, early intervention via gene therapy has significant potential to reverse or stabilize disease progression at early stages and prevent vision loss and the occurrence of more severe disease complications. ABO-503 is a novel AAV-vectored gene therapy that delivers wild-type *RS1* to photoreceptors using the AIM™ capsid AAV204. In this study, we examined RS1 expression specifically in photoreceptor cells via the *GRK1* promoter following subretinal administration in *Rs1* mutant mice. Animals were treated at p21 and examined 6 months later. Robust RS1 expression was observed in photoreceptor cells near the injection site as well as in the adjacent inner retina. RS1 expression was associated with an improvement in cone photoreceptor density and an increased thickness of the photoreceptor nuclear layer. In addition, full-field flicker ERG analysis showed a significant improvement in cone photoreceptor function. These experiments demonstrate that ABO-503 effectively rescues RS1 expression in mutant mice and delivers a measurable therapeutic benefit. We previously demonstrated that AAV204, expressing GFP, is superior to natural and other engineered capsids tested using intravitreal and para-retinal routes of administration. Additionally, para-retinal injection resulted in no observable immune response and robust expression in the macula, despite using a log lower dose. To eliminate the possible risk of irreversible damage from subretinal dosing in fragile retinoschisis eyes, further studies will assess delivery of ABO-503 via para-retinal administration in non-human primates to support future clinical development.

1132 Positive and Negative Selection of Anti-HIV-1 Gene-Modified Human Hematopoietic Stem/Progenitor Cells (HSPC) to Improve Engraftment and Efficient HIV-1 Inhibition with a Safety Kill Switch in Humanized BLT Mice

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Anti-HIV-1 HSPC-based gene therapy has great promise for HIV-1 cure. However, it's been difficult to achieve therapeutic levels of engraftment. Thus, a positive selection strategy is highly desirable for enriching the level of anti-HIV-1 gene-engineered cells for more robust HIV-1 inhibition. Additionally, the incorporation of a "kill-switch" to eliminate the genetically engineered anti-HIV-1 immune effector cells in cases of unexpected adverse effects, such as transformation by insertional mutagenesis and cytokine storm, is important for safety. We developed two positive selection strategies to maximize the repopulation of genetically engineered anti-HIV-1 gene-modified HSPC. First, we knocked down hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression using RNA interference that enabled us to effectively enrich anti-HIV-1 gene-modified HSPC using clinically available prodrug 6-thioguanine(6TG) in vivo. Second, we co-expressed truncated non-functional human epidermal growth factor receptor (huEGFRt) cell surface marker for rapid ex vivo sorting of gene-modified HSPC by FDA-approved anti-huEGFR monoclonal antibody Cetuximab. huEGFRt can be also used as a safety elimination strategy for the gene-engineered effector immune cells in case of unexpected adverse effects via cetuximab antibody-dependent cellular cytotoxicity (ADCC). We developed lentiviral vectors that express triple anti-HIV genes (CD4-based CAR, C46, and CCR5 shRNA) and either HPRTshRNA or huEGFRt for the selection strategies. The effectiveness of positive and negative selection and HIV inhibition of anti-HIV gene-modified human HSPC was examined in the humanized BLT (hu-BLT) mouse model. We successfully increased the engraftment of anti-HIV-1 vector transduced human CD34+ HSPC by knocking down HPRT expression and 6TG-mediated positive selection. Vector marking level (~1 vector DNA copies/cell) increased >2-fold over 7 weeks in hu-BLT mice (n=15, $p<0.001$). Viral load was significantly reduced >1-log reduction in 6TG treated mice compared to untreated mice (n=16) up to 4 weeks post-HIV-1 challenge ($p<0.05$). An anti-HIV-1 vector with huEGFRt resulted in >80% transduction in human CD34+ HSPC and transplantation resulted in high anti-HIV-1 vector marking at ~2 vector DNA copies/cell over 9 weeks in hu-BLT mice. The high level vector marking resulted in significant viral load reduction (> 0.76-log reduction $p<0.001$) for 6 weeks post-HIV-1 challenge compared to control non-transduced HSPC transplanted hu-BLT mice. We successfully reduced the huEGFRt expressing anti HIV-1 gene-modified cells by Cetuximab mediated negative selection for 15-fold in peripheral blood, 2-fold in spleen and 4-fold in bone marrow up to 5 weeks in hu-BLT mice. In conclusion, our newly developed positively and negatively selectable anti-HIV vectors have the potential to overcome the limitations of low engraftment of gene-modified HSPC for highly robust HIV-1 inhibition and an improved safety as a safety kill switch in cases of unexpected adverse effects caused by the genetically engineered cells.

1133 Using Design of Experiments to Optimize Microfluidic Transfection Platform for Cas9 RNP and mRNA Delivery in Various Cell Types

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Introduction: Mechanoporation can be used for intracellular delivery in which efficiency, cell health, and simple processing is needed. We have developed a microfluidic consumable designed to deliver gene

modifying payload into human primary cells using mechanoporation. Due to the large microchannel parameter space, thorough optimization for a specific application or cell types can require lengthy development times. We use a statistical approach of multivariate optimization using design of experiments (DoE) and response surface methodology (RSM) to assess the effects of several parameters and simultaneously optimize for maximizing transfection efficiency and cell viability. Using this approach, we can decrease the number of tests and the number of conditions necessary to find an optimal device parameter operating window. To test this approach, we delivered enhanced green fluorescence protein (EGFP) messenger RNA (mRNA) in hematopoietic stem/progenitor cells (HSPCs) as a proof of concept, a cell type with relevance in gene therapy with capabilities of self-renewal and differentiation into myeloid and lymphoid cell types. In addition to this application, we expand this approach to the transfection of either mRNA or Cas9 ribonucleoprotein (RNP) complex into fresh peripheral blood mononuclear cells (PBMCs) and CD3+ T-Cells subpopulations due to the clinical needs for T-cell engineering for cell therapy. **Materials and Methods:** Transfection was achieved by use of our Zephyr instrument to generate pressure-driven flow of cells and cargo through microfluidic consumables. The design of the experiment conducted was generated using JMP software to create a D-Optimal design for a response surface model. Analysis of the resulting data was also completed using JMP to fit a linear model and perform the subsequent stepwise-backward model reduction. A residual maximum likelihood model (REML) was constructed for each response to assess fixed device and instrument parameters. This method also allows us to include random effects, or effects that we would like to make inference about the wider population, rather than only the levels tested in this study. Delivery efficiencies were measured using flow cytometry to detect either the EGFP expression from mRNA, or the knock-out (KO) of T-cell receptor alpha/beta chain (TRAC) from the delivery of targeted Cas9 RNP. **Results and Discussion:** We assessed a list of microchannel design factors with potential to affect transfection efficiency and cell viability of HSPCs. We were able to achieve >80% mRNA delivery while maintaining >80% cell viability. By using DoE methodologies, we were able to quickly assess the importance of several device parameters and explore each factor's two-term interaction effects, as well as quadratic effects. Results of the follow up experiment confirmed model predictions within the 95% confidence interval that was created by the original model. After the success of this experiment for HSPCs, we have since discovered the optimal operating window of each parameter tested to simultaneously optimize both cell viability and transfection efficiency in the PBMC and CD3+ T-cell applications. **Conclusions:** In this study, we established an optimized microfluidic channel design that produced optimal mRNA delivery efficiencies and cell viability. We also expanded these methods to 2 additional cell types and an additional payload. The DoE methodology has simplified design search for complicated parameter space, requiring fewer runs to acquire quality data while assessing for statistical significance. In future studies, we plan to apply these methods to optimize for genetic modifications of HSPCs using CRISPR/Cas 9 RNP. Equal contributors: TD, IS, AK

1134 Optimizing Helper Plasmids by Removing Unnecessary "Junk" DNA Significantly Increases AAV Productivity

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Production of adeno-associated virus (AAV) vectors using live adenovirus infection has largely been replaced with a plasmid containing the necessary adenovirus genes to facilitate AAV replication for safety and logistical considerations. Helper plasmid (pHelper) contains three adenovirus genes: E2a, E4 and VA RNA. Although there are a number of different pHelper plasmids available, they all minimally contain these three genes, along with various additional sequence structure. These genes can contain larger regions than the promoter and coding sequence due to the nature of the adenovirus genome structure, that could be referred to as "junk" DNA as it pertains to AAV replication. We sought to reduce the size of our pHelper by removing regions of the adenoviral genes that were not essential to their function. Five plasmids containing various deletions were generated, with deletions ranging from 2-3.5 kb. All five of these smaller Helper plasmids increased vector genome (VG) productivity to various extents across multiple genomes. Capsid production also increases proportionally to VGs using the smaller pHelpers. We have achieved statistically significant increases in VG production through the removal of "junk" regions of our current Helper plasmid. Our working hypothesis is that the removal of non-coding regions of the adenoviral genes is altering the expression of E2a and E4 proteins, which enables higher capsid expression and in turn more VGs are packaged into the available capsids.

1135 False Positive HIV Testing Results and Recommendation for HIV Testing in Patients Treated with Lentiviral Based Chimeric Antigen Receptor T Cells

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Since the Food and Drug Administration first approved CD19-targeted chimeric antigen receptor (CAR) T cell therapy for B cell cancers in 2017, the number of products and indications have expanded, leading to an increasing number of cancer patients receiving CAR T cell therapy. To generate CAR T cells, cells isolated from patients are genetically engineered to express a CAR gene, which is often incorporated into a lentiviral vector. Most of the CAR T cell products are manufactured using a lentiviral vector with a modified HIV backbone and, thus, contains partial sequences from the HIV genome. Following CAR T cell infusion, patients treated with CAR T cells that contain the components of the HIV genome can have false positive HIV test results, which can cause unnecessary stress to the patient, families and caregivers. There are several approved clinical HIV diagnostic tests that use different technologies to detect HIV viral proteins or genetic sequences. Here we presented 10 patients as case studies for false HIV positive tests while receiving COH-19 CD19 CAR T therapy. Each patient was determined to be HIV negative prior to CAR T cell infusion and the

false positives were detected at various timepoints after CAR T infusion (6 months to 4 years). We then investigated the use of 3 different tests using PCR to detect HIV viral sequences including the long terminal repeat (LTR), group antigens (gag) and reverse transcriptase (pol). We collected peripheral blood from patients receiving commercial CAR T cell therapy, including tisagenlecleucel, lisocabtagene maraleucel, or axicabtagene ciloleucel which was used as negative control. We tested each of the post infusion patient samples for HIV using 1) COBAS AmpliPrep/COBAS TaqMan Quantitative HIV-1 test, 2) Aptima HIV-1 RNA Qualitative Assay, and 3) Abbott real time HIV test. We found that the COBAS TaqMan Quantitative HIV-1 test consistently yielded positive results in some patients post CAR T cell treatment. The COBAS TaqMan Quantitative test amplifies both LTR and gag sequences of the HIV viral genome, which are the parts of the sequence that are present in CAR T cells. As a result, this test may cause false positives in patients who have received CAR T cells. Neither of the other 2 tests resulted in positive results in samples collected from CAR T patients. Thus, when conducting HIV testing in patients with prior lentiviral based CAR T cell therapy, we recommend for patients with normal B cell counts to perform a HIV antigen/antibody combo test. For patients with B cell aplasia/hypogammaglobulinemia that an HIV PCR test is performed along with CAR specific persistent measurement such as quantitative WPRE detection. The COBAS TaqMan Quantitative test should not be used for HIV testing in patients receiving lentiviral based CAR T therapy.

1136 SNK-01 Autologous Enhanced Natural Killer Cells and an Immune Checkpoint Inhibitor Control Tumor Growth in Rare Chemotherapy-Resistant Advanced Soft Tissue Sarcomas

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Background and Rationale: Advanced or metastatic sarcoma is associated with an invariably fatal outcome. SNK01 is a first-in-kind, autologous non-genetically modified natural killer cell therapy with highly enhanced cytotoxicity and over 90% activating receptor expression which can be consistently produced from chemotherapy-treated patients. **Objective:** To report three unique rare cases of chemotherapy-resistant advanced soft tissue sarcoma (STS) who achieved durable partial responses and disease control with SNK01 plus an immune checkpoint inhibitor. **Methods:** Three patients with chemotherapy-resistant soft tissue sarcoma participated in FDA authorized and WIRB approved protocols. Cases #1 with desmoplastic small round cell tumor (DSRCT) received SNK01 (2 x 10^9 cells) and pembrolizumab 200 mg i.v. q 3 weeks. Cases #2 with radiation-induced chondrosarcoma (RIC) received SNK01 (4 x 10^9 cells) and pembrolizumab 200 mg i.v. q 3 weeks. Case #3 with undifferentiated spindle cell sarcoma was enrolled in the SK01-US01 study and received SNK01 (4 x 10^9 cells) i.v. q 2 weeks and avelumab 800 mg i.v. q 2 weeks. **Results:** Case #1(DSCRT): The tumors gradually decreased in size over one year to a 47% response. Patient then underwent a surgical debulking procedure followed by whole abdominal radiation and intraperitoneal chemotherapy, after which he resumed SNK01 and pembrolizumab regimen. His last scan showed no evidence of disease. He has now received 42 cycles of SNK01 + pembrolizumab over 38 months and has an Eastern Cooperative Oncology Group score of 0 (fully active, able to carry on all pre-disease performance without restriction. The reported median overall survival for advanced STS is 8-13 months. Case #2 (RIC): Patient had a 38% partial response after four months of treatment, underwent debulking surgery but died of post-surgical infection. The patient had received 18 cycles of SNK01 + pembrolizumab and survived 12 additional months. Case #3 (Undifferentiated spindle cell sarcoma): Patient had durable disease control (stable disease), has received 37 treatment cycles during the 17-month treatment period. The best reported median progression free survival for previously treated advanced STS is 4.1 months. Grade 3 or greater adverse events include hypothyroidism (n=1), increased ALT (n=1), increased alkaline phosphatase (n=1) and increased GGT (n=1) which were attributed to immune checkpoint inhibitor therapy. **Conclusion:** These unique case studies clearly demonstrate the potential of SNK01 in controlling tumor growth in conjunction with an immune checkpoint inhibitor with manageable toxicity. Further clinical trials need to be conducted to confirm these promising results.

1137 Acid Degradable Lipid Nanoparticles Efficiently Deliver mRNA

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The development of acid degradable linkages that rapidly hydrolyze at endosomal pHs is a central problem in the field of drug delivery and have been challenging to develop because of their instability. In this presentation we present a new class of acid degradable linkers based upon acetals that have an azide in their para position, which rapidly hydrolyze at endosomal pHs but have exceptional stability at pH 7.4 and solve the instability problems associated with using unstable acid degradable linkages. Azide-acetals hydrolyze via a two-step mechanism based upon reduction and acid hydrolysis, and this gives them a unique combination of stability and rapid triggerable hydrolysis. Azide acetals have a hydrolysis half-life of 129 hours at pH 7.4 and are stable enough to synthesize and incorporate into delivery vectors, however after in situ reduction with DTT they hydrolyze with a half-life of 17 minutes at pH 6.0 and rapidly degrade in endosomes. In this presentation, we used the azide-acetal linker to develop three new class of acid degradable lipids, consisting of PEG-lipids, cationic lipids and anionic lipids, which were significantly better at transfecting cells and mice than traditional lipids used in LNPs. Collectively, these experiments demonstrate the versatility of the azide-acetal linker and its potential as a platform for developing acid sensitive delivery vectors.

1138 Correlative Findings Following DSG3-CAART Infusion with and without Combination Preconditioning Therapy in Patients with Pemphigus Vulgaris (DesCAARTes Study)

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Mucosal-dominant pemphigus vulgaris (mPV) is a painful autoimmune blistering disease mediated by anti-desmoglein 3 autoantibodies (anti-DSG3 Ab). The current standard of care for mPV includes broadly immunosuppressive therapies that have risks of serious or life-threatening infection. We are evaluating the safety and activity of a novel cellular therapy consisting of gene-modified autologous T cells (DSG3-CAART) engineered to eliminate DSG3 reactive B cells in mPV. We previously reported on the translational and clinical data from mPV subjects receiving escalating doses of DSG3-CAART ranging from 1 x 10^5 to 7.5x10^9 transduced cells without preconditioning (NCT04422912). Manufactured DSG3-CAART eliminated target cell lines in vitro and were comprised of a mixture of effector and memory cells. DSG3-CAART cells were detected in the blood of all subjects within the first 29 days post-infusion with a dose dependent increase in peak persistence and persistence AUC for the first 29 days (AUC_{29d}), which reached a plateau at a dose of 2.5x10^9 DSG3-CAART cells. Here, we expand on those findings by including subjects who received combination therapy consisting of intravenous immune globulin (IVIG) to reduce potentially neutralizing autoantibodies and cyclophosphamide to reduce leukocytes followed by an infusion of 2.5x10^9 DSG3-CAART cells. Both peak persistence and persistence AUC_{29d} were further elevated in subjects receiving the combination therapy than those subjects receiving DSG3-CAART alone. Although persisting cells were predominantly of the central memory or stem cell memory phenotype in both groups, initial data from combination therapy subjects suggest that persisting DSG3-CAART cells exhibited signs of increased activation, including increased HLA-DR expression on DSG3-CAART cells, that were not observed in the DSG3-CAART cells in non-combination therapy subjects. Furthermore, in subjects receiving combination therapy, transient (< 2 weeks) leukopenia and neutropenia were observed but without lymphopenia. These data suggest that combination therapy with IVIG and cyclophosphamide enhances DSG3-CAART persistence and activation and support continued exploration of DSG3-CAART for mPV patients.

1139 Safety and Efficacy of EXG102-031, a Novel AAV-Based Gene Therapy for Neovascular AMD

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Age-related macular degeneration (AMD) is the most common cause of visual impairment among elderly. An estimated 20 million Americans aged 50 and older were living with AMD and patient numbers are predicted to increase with time. Approximately 10% of the AMD patients develop neovascular AMD, which is also called wet or exudative form of AMD (wAMD). EXG102-031, designed for the treatment of wAMD, is a recombinant adeno-associated virus (rAAV) expressing a fusion protein of angiopoietin 2-binding domain (ABD) and VEGF receptors (VEGFR) that is able to bind all known subtypes of VEGF as well as the angiopoietin 2 (Ang2). In the pharmacological studies, we first demonstrated that the fusion protein expressed from EXG102-031 was able to bind the three representative target ligands (VEGF-A, VEGF-C, and Ang2) *in vitro*. Furthermore, the efficacy of EXG102-031 for the treatment of neovascular AMD were comprehensively evaluated in laser-induced choroidal neovascularization (CNV) models (mouse and cynomolgus monkeys), as well as the *Tet/opsin/VEGF* double transgenic mice. The safety of EXG102-031 following a single subretinal injection of EXG102-031 was assessed in a GLP-compliant toxicology and biodistribution study in cynomolgus monkeys. EXG102-301 demonstrated strong *in vivo* efficacies in dose-range finding (DRF) studies in three different AMD models, laser-induced CNV in NHP, laser-induced CNV in mouse, and the *Tet/opsin/VEGF* double transgenic mice. It is worth to note that in the DRF study in *Tet/opsin/VEGF* double transgenic mice, the total retinal detachment was reduced by 80% after the injection of 1×10^9 vg and 100% after the injection of 7×10^9 vg/eye of EXG102-031. Mice in the positive control group received AV8-antiVEGFfab vector (expressing Ranibizumab) at a dose of 5×10^9 vg/eye showed an inhibitory effect on retinal detachment comparable to that found in the 1×10^9 vg/eye dose group received EXG102-031. The safety of EXG102-031 delivered via a single subretinal injection was comprehensively evaluated in a GLPcompliant toxicology study in NHP. EXG102-031 was well tolerated and did not cause any apparent systemic toxicity following a single subretinal injection at doses of 2×10^{10}, 1×10^{11}, and 5×10^{11} vg/eye in NHP. Subretinal injection induced transient and reversible ocular inflammation in the early phase including in the Vehicle control group. Following subretinal administration, EXG102-031 was mainly distributed in the ocular tissues of NHPs. Except for a low level of vector genomes detected in spleen, epididymis, liver, bladder lymph nodes and lung in a subset of the animals, vector genome in remaining tissues of most animals was not detected. Taken together, these nonclinical pharmacology and toxicology studies strongly support clinical development of gene therapy with EXG102-031 for treatment of patients with neovascular AMD. A phase I clinical study is planned to evaluate the safety and tolerability of EXG102-301.

1140 A Lentiviral Vector-Based Gene Therapy Product for the Treatment of Wilson Disease

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Wilson Disease (WD) is an inborn error of metabolism caused by mutations in a copper transporter ATPase copper transporting beta polypeptide (ATP7B). This results in impaired function of the protein and accumulation of copper in organs, primarily the liver but also in the brain and the eye. Untreated, the accumulation of copper in these organs causes damage and can result in liver failure. The current treatments for WD, metal chelators, aim to remove excess copper, reducing intracellular copper and preventing its re-accumulation. Although the use of systemic copper chelators is relatively successful in controlling symptoms, the costs of these life-long therapies is very high, the side effects can be intolerable to many patients resulting in reduced compliance, and ultimately the underlying disease mechanism remains unresolved which may require liver transplantation. Gene therapy offers the opportunity to treat WD patients by providing a corrected copy of the gene and removing the necessity for life-long medication. The ATP7B gene is 4.4 Kb long and beyond the capacity of AAV vectors. To solve this issue, the size of the open reading frame can be reduced, or an alternative vector system can be used. Lentiviral vectors (LVV) have the capacity to accommodate the full length ATP7B gene. LVV also have the advantage of integrating into the host genome and providing long-term stable transgene expression from a single administration. This is of particular importance when targeting organs such as the liver, which have a high turnover rate especially in paediatric patients; in these patients, use of an AAV vector would result in loss of expression to below therapeutic levels as the cells divide over time. A HIV vector encoding human ATP7B using a hepatocyte-specific promoter was evaluated in a WD cell model *in vitro.* This vector demonstrated cell protection from copper induced toxicity. Transgene expression was sustained *in vitro* over 31 days. This vector was then evaluated in a juvenile Atp7b-/- mice, a model for WD. Analysis of the relevant biomarkers and survival will be presented. We have demonstrated efficacy *in vitro* and *in vivo* with an HIV vector expressing full length ATP7B which has the ability to stably integrate and provide long term gene expression following a single administration. This efficacy data taken together with Oxford Biomedica's ability to produce high quantities and quality of clinical and commercial grade vector provides a robust approach to treat Wilson Disease.

1141 Automated Manufacture of dNPM1 TCR-Engineered T Cells for Therapy

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Treatment of relapsed or refractory acute myeloid leukemia (AML) still represents a challenge for physicians and is mainly associated with poor prognosis with a 5-year survival rate in Europe of only 25%. A neoantigen encoded by mutated nucleophosmin 1 (dNPM1) is expressed in approximately 30% of patients and presents an attractive target for immunotherapy with T cell receptor (TCR)-engineered T cells. Manufacturing of TCR-modified T cells, however, is still limited by a complex, time-consuming and laborious procedure. Therefore, this study specifically addressed the requirements for a scaled manufacture of dNPM1-specific T cells in an automated, closed and Good Manufacturing Practice (GMP)-compliant process using the CliniMACS Prodigy. Small scale studies demonstrated that the HLA-A*02:01 restricted recombinant TCR is dependent on CD8 co-receptor interaction to bind the peptide-HLA complex with high affinity. We therefore focused on the development of a CD8 T cell-specific process. Thus, in 23 individual runs, 2E8 CD8-positive T cells were enriched from cryopreserved leukapheresis, subsequently activated, lentivirally transduced, expanded and finally formulated. By adjusting and optimizing culture conditions, we additionally reduced the manufacturing time from twelve to eight days while still achieving a clinically relevant yield of up to 5.5E9 dNPM1 TCR-engineered T cells (4.2E9 ± 1.4E9). The cellular product mainly consisted of highly viable CD8-positive T cells (97.5% ± 0.978%) with an early memory phenotype. The cellular product was stable for up to 48h. Overall, mean vector copy number was 2.9 ± 1.1. Importantly, TCR-engineered T cells specifically lysed dNPM1-positive target cells *in vitro* as well as *in vivo*. In conclusion, we demonstrated that our CD8 process is robust and reproducibly yielded suitable numbers of potent dNPM1 TCR-engineered T cells, preparing the way for initiating a clinical trial to treat patients with relapsed or refractory AML.

1142 Immune Response to AAV Vector Capsid and Assessment of Eligibility for AAV-Mediated Gene Therapy for Duchenne Muscular Dystrophy

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Despite advances in AAV gene therapy, the field still faces a crucial unresolved challenge of the host immune response against vector capsid proteins. We hypothesize that our proposed immunosuppression

regimen can reverse humoral and cellular responses in mice with pre-existing immunity for AAV and will allow safe AAV9-μDysUF administration and increase dystrophin expression in mice. We have developed an optimal microdystrophin (AAV9-μDysUF) that improves exhaustion and *in vivo* and *ex vivo* muscle force compared to μDys5* and GFP-injected *mdx* mice. Utilizing the wildtype C57BL/6 mouse, we injected empty AAV9 capsids in mice to mimic pre-existing immunity in human patients. We collected serum samples from mice on Day 0 before injection. The mice were then injected with empty capsids at a dose of $1X10^{12}$ vg/kg on day 0 to induce an approximate level of anti-AAV antibodies of 150U/ml. Four weeks later, after collecting serum, the same animals (N = 6) received different immunosuppression drugs, either alone or in combination with other drugs. The immunosuppression drugs are velcade, darzalex, anti-CD20 antibody, and sirolimus. The animals were maintained in these drug regimens until day 70. On day 70, serum was analyzed from these groups. We measured the anti-AAV titers at days 0, 22 and 70 and compared the respective time points to animals with no immunosuppression. We found a **significant decrease in AAV antibody titers** between no immunosuppression and animals on immunosuppression drugs. At day 70, sera from animals treated with antiCD20 antibody+sirolimus show 33%, velcade show 60%, darzalex show 60%, antiCD20 antibody+sirolimus+velcade show 88% and antiCD20 antibody+sirolimus+darzalex show 51% reduction in anti AAV9 titer respectively. This decrease in anti-AAV9 titer is significant compared to no immunosuppression group. Furthermore, we plan to implement the best immunosuppression regimens for safe AAV9-μDysUF administration and increase dystrophin expression in mice. We are implementing immunomodulation strategies like an induction regimen before a high dose (1E14vg/kg), two low doses (5E13vg/kg), a maintenance regimen of anti-CD20 antibody, and sirolimus during the study to analyze which dose will provide optimal microdystrophin expression.

1143 Transitioning from Adherent to Optimized and Scalable Suspension-Based Production of CAR-Lentivirus (LV)

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The number of lentiviral-based chimeric antigen receptor (CAR) T cell therapies in development pipelines continues to grow substantially. Lentiviral vectors (LVV) have proven to be an efficient mechanism for the delivery of genetic material to T cells. However, depending on the strategy, the production of these vectors can come at a high cost. While adherent cell lines, such as HEK293T cells grown in static culture, have traditionally been the first choice for LVV production, the scalability and automation of these approaches is limited and costly. Conversely, suspension-adapted cell cultures grown in stirred-tank bioreactors can scale to volumes upwards of thousands of liters in a single batch. Unlike adherent cells, suspension-based cultures aren't serum-dependent, which makes them a more appealing candidate for commercial manufacturing. The aim of this work was to optimize the transient-transfection conditions for a preclinical CAR construct and scale the process to meet anticipated manufacturing needs for clinical trials. A Good Manufacturing Practice (GMP)-compliant HEK293 suspension cell line was chosen for this process. Previously, the optimal viable cell density (VCD) at transfection, total amount of DNA, and other conditions were determined using a Green Fluorescent Protein (GFP) construct. Using a Design of Experiments (DoE) approach, a series of three iterative transfection experiments were conducted at small-scale. For each experiment, two factors were varied: the percentage of CAR transfer plasmid relative to total DNA and the ratio of the packaging plasmids. Scale-up in 1 L shake flask cultures was completed using the top two performing conditions identified in the DoE, followed by bench-scale demonstration in bioreactor cultures. Baseline production for this CAR-LVV in an adherent HEK293T cell line was determined to be 2×10^7 TU/mL. After optimization, the suspension-based upstream process consistently yielded between 1×10^8 to 1.2×10^8 TU/mL.

1144 Intratumoral Delivery of CRISPR-Cas9 Using Lipid Nanoparticles Enables Efficient Gene Editing and Restores Chemosensitivity in Lung Squamous Cell Carcinoma

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Recent studies point to the evolution of drug resistance in non-small-cell lung cancer (NSCLC) as being centered, at least in part, on the upregulation of various genes involved in controlling efflux and drug metabolism. Among the most important of these genes is Nuclear Factor Erythroid 2-Related Factor (NRF2), considered the master regulator of over 100 target genes involved in cellular responses to oxidative and/or electrophilic stress. We are utilizing a combinatorial approach in which CRISPR-directed gene editing is used to disable NRF2 in order to augment standard-of-care chemotherapy treatment. We developed and optimized lipid nanoparticles (LNP) to deliver Cas9 mRNA and a single guide RNA (sgRNA) targeting mutant NRF2, as an intratumoral injection. We show our single guide RNA distinguishes the genome of a tumor cell and a normal cell, thereby conferring target selectivity onto this therapeutic approach. We also show efficient gene editing activity after intratumoral LNP-mediated delivery of CRISPR/Cas9 in vitro and in vivo, with limited biodistribution and low off-target profile. We will discuss our progress in developing this CRISPR-based gene therapy.

1147 Enhanced CCNG1 Expression in Tumors May Predict Clinical Benefit from DeltaRex-G, a Tumor Targeted Retrovector Encoding a CCNG1 Inhibitor Gene

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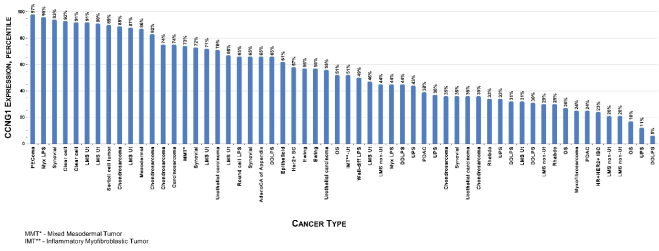
Background: Metastatic cancer is associated with an invariably fatal outcome. Therefore, innovative therapies are urgently needed. Although expanded access for DeltaRex-G, a tumor targeted retrovector encoding a CCNG1 inhibitor gene, is on-going for an intermediate-size (n= up to 40) population of advanced sarcoma and pancreatic cancer, more data is needed to identify patients who are likely to benefit from DeltaRex-G gene therapy. In this study, we retrospectively analyzed CCNG1 expression in archived tumors of patients who were previously treated with DeltaRex-G and who are active candidates for DeltaRex-G therapy. **Methods:** Archived formalin-fixed paraffin embedded (FFPE) tumor specimens (n=58) from patients with solid malignancies who are actively followed at the Cancer Center of Southern California were collected, processed, and subjected to RNA sequencing. Briefly, RNA-seq libraries were sequenced to generate 50 million reads that were aligned using Kallisto v0.42.4 to GENCODE v23 transcripts with default parameters. Only protein-coding, IGH/K/L- and TCR-related transcripts were retained for downstream processing, resulting in 20,062 protein coding genes. Gene expression was quantified as transcripts per million (TPM) and log2-transformed. A gene expression level is presented as low/medium/high depending on the expression level of a such gene in patients of the reference cohort. Low = <17%; Medium = 17%-83%; High = >83%. **Results:** Thirty-two male and 26 female subjects, ages ranging from 16 to 86 years were studied. Forty-nine (84.4%) patients had sarcoma, 3 (5.2%) had urothelial carcinoma, 2 (3.5%) had breast carcinoma, 2 (3.5%) had pancreatic cancer, 1 (1.7%) had Sertoli cell tumor, 1 (1.7%) had adenocarcinoma of appendix. Eleven (19%) tumors showed high CCNG1 expression, 44 (76%) tumors had medium expression, and 3 (5.2%) tumors had low CCNG1 expression. Of note, the tumor of one 14-year survivor with metastatic pancreatic adenocarcinoma in sustained remission had 24 % CCNG1 expression, one 3-year survivor with metastatic chondrosarcoma metastatic to lung with stable disease had 74% CCNG1 expression, one 2-year survivor with early stage HR+ HER2+ breast cancer in remission had 23% CCNG1 expression, and one 2-year survivor with early stage triple negative breast cancer in remission had 74% CCNG1 expression. **Conclusion:** Taken together, these data indicate that (1) Medium to high CCNG1 expression was found in 95% of tumors studied, and (2) Patients with medium CCNG1 expression who received DeltaRex-G had clinical benefit and are alive in sustained remission or with stable disease 2-14 years from DeltaRex-G treatment initiation, and (3) Prospective studies are warranted to correlate CCNG1 expression level and response to DeltaRex-G therapy.



1148 An Automated, Closed Magnetic Cell Separation System for Both Autologous and Allogenic Cell Therapy Development

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Adoptive cell therapy has proven clinical efficacy in many cancer types and the success has driven more development of new drugs. However, on the manufacturing side, current process using conventional tools is complicated and results in suboptimal purity and yield sometimes. All those shortcomings may contribute to the high cost of the cell products and limit the patients access to drugs. Cell therapy developers in both industry and academic settings are trying to improve their manufacturing process aiming to increase efficiency and lower cost. Cell selection done with magnetic beads antibody conjugate is an important step in cell manufacturing: it is either the first step to isolate T cells for gene modification or the last step to deplete subtype of T cells in allogenic transplantation, so a fast efficient magnetic cell separation system has been desired. We have developed an automated closed system (MARS® BAR) based on Applied Cells proprietary column-free matrix-free in-flow magnetic cell separation technology. The system can deal with diverse cell sources without requiring cell washing before or after labeling, thus it makes the cell selection workflow very simple. The MARS® BAR system has no capacity limit and is easy to scale-down or scale-up, which make it suitable for both autologous and allogenic cell therapy manufacture. Here we present the results of CD4/CD8 T cell isolation from fresh leukopak using MARS® BAR and its closed fluidic kit. Cells were labeled with CD4 and CD8 nano magnetic beads in the bag and diluted in the bag before the magnetic separation. The positive selection program ran through up to 120million cells per minute and resulted in >90% purity and >85% recovery of T cells. In the followed cell activation and expansion, we proved the unaffected viability and functionality of the isolated T cells. We also present the results of TCR αβ+ cell depletion from PBMC to demonstrate the capability of purifying cells in allogenic cell therapy manufacturing. In the automated process cells were only passing the magnetic separation channel once and 99.9% depletion of TCR αβ+ cells with 90% recovery of TCR γδ cells was achieved. The magnetic cell selection using MARS® BAR automated closed system shortens the process time and increases the yield of target cells comparing to conventional tools therefore it shows the potential to help lower the manufacturing cost.

The system is built for a GMP-compliant environment and provides a new solution for cell selection in both autologous and allogenic cell therapy development.

1149 Reversion of Non-Alcoholic Steatohepatitis by Skeletal Muscle-Directed FGF21 Gene Therapy

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Non-alcoholic steatohepatitis (NASH) is a severe form of nonalcoholic fatty liver disease, which is driven by the global obesity and type 2 diabetes epidemic. NASH is characterized by liver inflammation and fibrosis that can progress to cirrhosis, liver failure, cancer and death. Currently, NASH is an unmet medical need. Here, we demonstrated that adeno-associated viral (AAV) vector-mediated-FGF21 genetic engineering of skeletal muscle of high-fat diet-fed obese and insulin-resistant mice resulted in increased circulating levels of native FGF21. AAV-FGF21-treatment normalized liver fat content, liver inflammatory and fibrotic markers and counteracted hepatocyte damage, consistent with NASH resolution. It also precluded progression from NASH to cirrhosis and hepato-cellular carcinoma. Moreover, circulating FGF21 reversed adipose tissue hypertrophy and inflammation, increased energy expenditure, normalized glycemia and insulinemia, and increased insulin sensitivity. Scale-up of muscular FGF21 gene transfer to large animals also successfully resulted in long-term secretion of biologically active FGF21 and therapeutic effects in key target tissues, in the absence of adverse events. All these results point out the potential of the AAV-FGF21-mediated gene therapy to treat NASH and support the clinical translation of the approach.

1150 Targeted Exon Skipping of DMD Exons 51 and 53 Using PAM-Less Base Editors

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Duchenne Muscular Dystrophy (DMD) is a devastating X-linked monogenic disease caused by mutations in the *DMD* gene that prevent production of a functional dystrophin protein leading to progressive skeletal and cardiac muscle degeneration, and ultimately death. Exon skipping has emerged as a promising treatment modality for many DMD cases by circumventing disruptive mutations and restoring the reading frame of the dystrophin gene to drastically improve the symptoms of the disease. While the FDA approved antisense oligonucleotides (AONs) for exon skipping, they require repeated injections to achieve a mild effect and their cost is very high. Alternatively, CRISPR-Cas9 has revolutionized the field of genome editing and has been harnessed for exon skipping through disruption of splice sites by creating targeted double strand breaks (DSBs). While Cas9 mediated exon skipping of various exons within *DMD* has been successfully demonstrated, the drawbacks of creating DSBs, such as chromosomal translocations, unpredictable editing outcomes, and a TP53 DNA damage response that compromises the survival of edited cells, severely limit this approach. To overcome these limitations we have previously developed a gene editing platform termed CRISPR-SKIP that uses CRISPR-Cas9 single base editors to achieve permanent exon skipping by directly mutating splice sites within the DNA without creating a DSB. Although this technique can be applied to skip many exons, the availability of an appropriately positioned protospacer adjacent motif (PAM) sequence that places the targeted base within the editing window of the BE can prevent the targeting of exons that are therapeutically relevant. Here we demonstrate the use of an engineered PAM-less SpRY-Cas9 base editor coupled to an evolved ABE8e deaminase domain to target and edit the splice sites of *DMD* Exons 51 and 53, the two exons that when skipped, would treat the largest DMD patient populations but whose splice acceptors have been resistant to editing with wild-type Cas9. We first systematically optimized our CRISPR-SKIP approach for these targets by testing a large panel of single guide RNAs targeting the splice acceptors and splice donors to identify the guides that maximize editing rates of these sites. We next developed a novel human myoblast DMD disease model harboring a deletion of exon 52 which can be corrected through skipping of exon 51 or exon 53. This cell culture model was then used to evaluate the DNA editing efficiency and exon skipping rates following delivery of our optimized CRISPR-SKIP system, and to evaluate the restoration of dystrophin protein expression after differentiation into muscle tissue in a 3D scaffold. This work provides valuable preclinical data on the use of CRISPR-SKIP as a therapy for DMD, and demonstrates the enormous therapeutic potential of using PAM-less base editors to treat human disease.

1151 CAR T Cell Production Expands Endogenous T Cell Repertoire Recognizing Additional Tumor Antigen(s)

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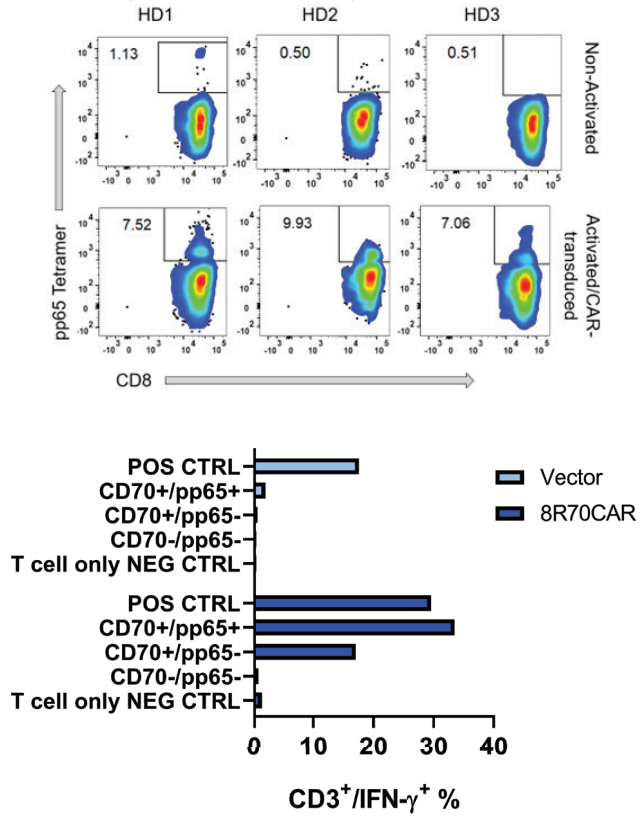
BACKGROUND: Glioblastoma (GBM) is an aggressive and heterogeneous brain tumor that currently has no curative treatment. Our group identified CD70, which is overexpressed by low-/high-grade gliomas and associated with poor patient survival. We established a CAR-T cell therapy platform targeting CD70-expressing gliomas, which showed efficient antitumor activity preclinically. Modification of CD70CAR to express IL-8 receptors (8R-70CAR) enhanced tumor trafficking and persistence, resulting in improved antitumor efficacy and long-lasting immunity to tumor rechallenge. These findings culminated in a phase I trial of 8R-70CAR T cells (NCT05353530) for patients with newly diagnosed GBM that will begin this year. However, CAR T cell therapy currently faces obstacles such as single antigen targeting in heterogeneous tumors and maintenance of activation of CAR T cells. Cytomegalovirus pp65 protein is a tumor-specific target in GBM. A combinatorial approach against CD70 and pp65 may result in enhanced effect against heterogeneous GBM. **OBJECTIVE:** Generate and test CD70 and pp65 dual-target T cell therapy against GBM. **METHODS:** Healthy donor peripheral blood mononuclear

cells were stimulated with anti-CD3/CD28 Dynabeads and retrovirally transduced with 8R-70CAR. Frequencies of 8R-70CAR- and pp65-specific T cells were evaluated by flow cytometry. **RESULTS:** The pp65-specific CD8+ T cell population was expanded post DynaBeads activation to a frequency tenfold over baseline (Fig. 1). Single-specific (CD70 CAR+ only and pp65-specific only) and double-specific (CD70 CAR+ and pp65-specific) T cells were successfully generated. Importantly, these cells secreted greater IFN-g when tumor cells expressed both targets (Fig. 2). **CONCLUSION:** The CAR T cell production process significantly expands pp65-specific T cells, resulting in enhanced antitumor efficacy through recognition of both tumor targets. Our CAR T cell product may contain T cell repertoires that effectively target multiple tumor antigens.



1152 G-CSF-Free HSC Mobilization with WU-106/AMD3100 Allows for Safe and Efficient *In Vivo* HSC Transduction and *In Vivo* Prime Editing of the Sickle Cell Disease Mutation in a Mouse Model

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We have previously developed a new *in vivo* HSC gene therapy approach that does not require myeloablation/conditioning and HSC transplantation. In this approach, HSCs are mobilized from the bone marrow into the peripheral blood stream and transduced with intravenously injected helper-dependent adenovirus (HDAd5/35++) vectors that target human CD46, a receptor that is abundantly expressed on primitive HSCs. HSCs transduced in the periphery return to the bone marrow and spleen and persist there long-term. The approach has recently been used for *in vivo* editing of HSPCs to correct the Sickle Cell Disease mutation in erythroid cells of CD46/Townes mice ($CD46^{+/+}$/ hα/hα::β^S/β^S triple homozygous mice) a mouse model for SCD. In that study, we mobilized cells by injection of G-CSF for 5 days followed by one injection of the CXCR4-inhibitor AMD3100. G-CSF treatment is known to trigger leukocytosis and release of pro-inflammatory cytokines from granulocytes and is thought to cause critical toxic side effects in SCD patients. Recently, we developed a simpler, rapid, and more HSC-selective mobilization regimen using truncated GRO-β (MGTA-145), a CXCR2 agonist, and plerixafor/AMD3100. We demonstrated that it mediated efficient mobilization and *in vivo* transduction of HSCs with β-thalassemia correction in mice. Among the alternative G-CSF-free mobilization protocols is also plerixafor plus small molecule VLA4 antagonists, such as BIO5192 or its improved derivatives (e.g. WU-106). Here we tested WU-106 plus AMD3100 mobilization for *in vivo* HSC transduction with a SCD prime editing vector (HDAd-PE5max) in CD46/Townes mice (N=7). Two hours after subcutaneous injection of WU-106 + AMD3100, mice received one intravenous injection of HDAd-PE5max. To expand edited cells, *in vivo* selection with low-dose O^6BG/BCNU was implemented on days 6, 19, and 33 while the episomal HDAd vector was still present in HSPCs. Mobilization with WU-106 + AMD3100 in CD46/Townes was efficient (and comparable to the G-CSF/AMD3100 regimen) as reflected by the presence of (on average) 10^5 $Lin^-/Sca1^+/c\text{-}kit^+$ (LSK) cells and 10^4 colony forming units (CFU) per ml peripheral blood at 3 hours after mobilization drug injection. In contrast to the GCSF/AMD3100 approach, leukocytosis was minimal and serum cytokines (IL-6, TNF, IFN-γ) were not detectable after WU-106+AMD3100. SCD target correction was measured in PBMCs by Sanger sequencing. At week 6 after *in vivo* transduction, editing rates at the T>A site (SCD repair) site were in the range of 15 to 33% in the seven mice (average 20%). Prime editing is independent of double-strand DNA breaks, and we have shown that *indels* on average were less than 1%, which is important for editing a coding region. Mice will be followed for 16 weeks and then HSCs will be transplanted into secondary recipients. Analysis of the percentage of β^S versus β^A globin in erythrocytes and phenotypic correction in primary mice (spleen size, histology, blood smears) will be reported. These first data indicate that HSC *in vivo* gene therapy using the new mobilization approach is safer than GSCF/AMD3100, can be done on one day, and can achieve therapeutically relevant correction levels of the SCD mutation in a relevant mouse model.

1153 The reiMMAgine Study: Evaluation of the Safety, Tolerability, and Pharmacodynamics of AAV8 Gene Therapy (MMA-101) in Combination with an Immunotolerance-Inducing Nanoparticle (ImmTOR) in MUT Subtype Isolated Methylmalonic Acidemia (MMA)

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MMA due to methylmalonyl-CoA mutase (MMUT) deficiency manifests as episodic metabolic decompensation with progressive decline in multiple organ systems. In severe patients, dietary and medical interventions fail to prevent metabolic instability and liver transplant is needed in some patients to reduce hospitalizations and improve survival. Liver directed gene therapy may present a viable alternative to liver transplantation for these patients. The reiMMAgine study has been designed to evaluate safety and efficacy of a systemic AAV8 gene therapy designed to express a liver targeted, wild-type *MMUT* (MMA-101; hAAT promoter, apoE enhancer) in *mut*^{0} MMA subjects. The study is an open-label, single dose level, single center (NIH) study of MMA-101 plus the immunomodulatory drug ImmTOR, which is nano encapsulated sirolimus. Previously we demonstrated in adult volunteers that ImmTOR inhibited the formation of anti-AAV8 neutralizing antibodies (NAb) following administration of AAV8 empty capsid in a dose-dependent manner at Day 30, but most subjects had an increase in NAb by day 90 [MOL THERAPY 2022:336-337)]. Therefore, eligible subjects will receive IV ImmTOR prior to the IV dose of MMA-101, as well as ImmTOR alone on days 28 and 56, for the purpose of mitigating long-term development of NAb. In addition, ImmTOR may enhance transgene expression as observed in animal studies and improve the safety of the gene therapy by ameliorating the cellular immune response-mediated liver injury and potentially reducing the need for chronic use of steroids and other immunosuppressants. Enrolled subjects will have a molecular genetic diagnosis of MMUT MMA AND serum methylmalonic acid (MMA) levels between 100 to 3,000 μmol/L AND a clinical history consistent with severe MMA by agreed criteria with the FDA. The study will treat up to a total of 6 subjects, 3 adolescents (≥12 and <18 years of age) in Cohort 1 followed by 3 children (≥3 and <12 years of age) in Cohort 2. MMA-101 will be administered at a dose of 1.0E13 vg/kg. The first subject in Cohort 1 of adolescent subjects will be treated with MMA-101 alone, with the addition of steroid prophylaxis. The second adolescent subject in Cohort 1 will be treated with IV of ImmTOR followed by MMA-101 on Day 1 and then ImmTOR on Days 28 and 56, without addition of steroids. The dose of ImmTOR in the third subject in Cohort 1 may be increased depending on immunologic assessment of anti-AAV8 NAb, ImmTOR pharmacokinetics, and safety in the second subject. Following safety and efficacy assessment of Cohort 1, Cohort 2 will be conducted in children (≥ 3 and <12 years old) in the same fashion, omitting the use of steroids in all three subjects. The primary endpoint for assessment of safety and efficacy will be one year following MMA-101 infusion, with interim assessment at 3 months to assess proceeding with next subject and/or escalating to the next cohort. All subjects will be followed for an additional 4 years. The endpoints include: 1) Safety and tolerability; 2) Pharmacodynamic response to MMA-101 assessed by reduction sMMA and an increase in the 1-13C -sodium propionate oxidation breath test; 3) Formation of AAV8 neutralizing antibodies (NAb); 4) clinical parameters including hospitalizations, metabolic crises, growth, diet, and subject-/caregiver-reported outcomes. In summary, this first-in-human clinical trial will establish the safety and efficacy of a liver-directed AAV8 gene therapy to treat subjects with *mut*^{0} MMA and assess the ability of the novel immunomodulatory drug ImmTOR to induce immune tolerance to the AAV8 vector.

1154 Development and Qualification of 4-plex Assay for AAV Vector Genome Integrity Evaluation by dPCR

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The production of viral vectors for gene therapy is focused on pure, safe, and efficacious products. Absence of impurities and presence of full vector genomes play a crucial role. We have addressed the problem of AAV vector genome integrity by developing an advanced dPCR multiplex approach. The newly developed 4-plex assay was qualified on DNA level and tested on several different AAV vectors to show its applicability. The assay is an upgrade from simplex vector genome titering assay by providing accurate quantitative result on genome integrity. The use of such assay can better guide process development with a goal of having as much full vector genomes as possible.

1155 Long-Term Seroconversion Risk Among a Cohort of Cynomolgus Macaques

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AAV drug development has been aided by use of preclinical animal models to assess vector safety and efficacy. Among preclinical species, non-human primates have been an effective large animal model to interrogate drug efficacy and toxicity. However, AAV vector administration is ineffective in animals carrying neutralizing antibodies from a previous exposure. Such antibodies bind and neutralize vector resulting in reduced efficacy, modified biodistribution, and increased toxicity. Hence, only seronegative animals are included in AAV preclinical studies. Seronegativity is determined by prescreening animals for anti-AAV antibodies using either a cell-based neutralizing antibody (NAb) assay or an ELISA-based binding antibody (BAb) assay. However, even NAb negative animals risk seroconversion while awaiting study start which can led to reduced gene transfer. Here, we undertook a longitudinal study over 6 months to assess seroconversion risk among a cohort of cynomolgus macaques. Animals were prescreened and twelve AAV9 seronegative macaques (6/gender) were procured, shipped, and housed (2/cage) before study start. Several guidelines were implemented to reduce environmental

exposure while animals were housed. Animals were bled monthly, and serum investigated for anti-AAV9 antibodies. While animals were NAb negative before shipment, two out of the twelve animals (CIC018; female and CHE018; male) were AAV9 NAb seropositive (titers 5 and 40, respectively) before study start. Animals were positive for the study duration with no fluctuations in titer. Cage mates co-housed with the seropositive animals did not seroconvert suggesting a lack of transmission. BAb were marginally higher in the animal with higher NAb titer. The seroconversion of two animals to AAV9 led us to investigate NAb and BAb against a panel of AAVs belonging to different clades (AAV1, 2, 3B, 5, 6, and 8). Animal CHE018 had cross-reactive NAb to AAV8, albeit at lower levels, while CHF050 had higher NAb to AAV3B, and lower cross-reactive titers against AAV1 at all time points. IgM binding antibodies were higher in one animal (CIC023) against all AAVs at study start that declined below detection levels by 2 months. Surprisingly, there was no concordant increase in IgG levels against any AAV in this animal. Similarly, IgG levels peaked in two animals after 4 months without an expected prior increase in IgM levels. These findings suggest the risk of seroconversion of a naïve animal co-housed with a seropositive cage mate appeared to be low. Finally, while NAb titers were more stable, IgG and IgM binding antibodies fluctuated more during the study and were less predictive of seropositivity.

1156 Durability of AAV-Mediated Phenotypic Correction of Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) Deficiency in Mice

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Previously, the authors presented results of modified adeno-associated virus (AAV)-ACADVL (gene encoding human very long-chain acyl-CoA dehydrogenase [VLCAD] protein) vector constructs that showed proof-of-concept in VLCAD$^{-/-}$ mice up to 8 weeks post-dose. Here we present data for up to 24 weeks post-dose. VLCAD deficiency is a rare, autosomal-recessive disorder of fatty acid oxidation with an incidence rate from 1:40,000 to 1:120,000 (about 50-100 babies born per year in the US). Mutations in the *ACADVL* gene lead to reduced levels of VLCAD, an inner-mitochondrial membrane protein, which catalyzes the rate-limiting step in β-oxidation. The most severe form of VLCAD deficiency (i.e., early or neonatal-onset), generally presents with hypoketotic hypoglycemia, hepatomegaly, and cardiomyopathy that can be fatal if not diagnosed early. While dietary management with medium-chain triglyceride oils has proven beneficial in preventing some symptoms, the risk of metabolic decompensation remains throughout a patient's life, particularly during times of increased energy demand. Additional durability data show continued gene expression of AAV9-ACADVL in VLCAD-deficient mice for up to 24-weeks post-dose. VLCAD$^{-/-}$ mice were treated systemically with various doses of two lead AAV9-ACADVL constructs and subjected to a cold-fast challenge at Weeks 12 and 24. This disease-specific phenotype was assessed by monitoring the animal's ability to thermoregulate and maintain euglycemia after a fasting cold challenge. Similar to results observed at 8-weeks post dose, internal body temperatures of untreated VLCAD$^{-/-}$ mice dropped below 20°C and animals were hypoglycemic, resulting in mice becoming lethargic and moribund. In contrast, WT mice and AAV9-ACADVL-treated VLCAD$^{-/-}$ mice were able to maintain body temperatures and euglycemia. VLCAD protein expression in target tissues (liver, cardiac muscle, and skeletal muscle) was verified with IHC staining, Western Blot analysis, vector copy numbers and mRNA expression. Overall, the results from this study demonstrate the long-term durability of an AAV-mediated gene therapy to correct key biochemical and phenotypic aspects of VLCAD deficiency.

1157 Non-Canonical Amino Acid Incorporation into AAV5 Capsid Enhances Lung Transduction in Mice

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Although recombinant adeno-associated virus (rAAV) is the leading in vivo delivery vehicle for gene therapy, efficient transduction of certain tissues, such as the lung, remains a challenge. Novel AAV capsids usually carry peptide substitutions and/or insertions that comprise genetically encoded natural amino acids. In this study, we utilized genetic code expansion to incorporate Nε-2-azideoethyloxycarbonyl-L-lysine (NAEK), a non-canonical amino acid (ncAA), into the variable regions (VRs) of AAV5 capsid, and found that NAEK-modified rAAV5 exhibited enhanced lung tropism in mice. First, we incorporated NAEK into the AAV5 capsid at individual sites using an orthogonal suppressor tRNA system. Five rAAV5.NAEK mutants packaging the same EGFP transgene cassette were purified and evaluated for tissue tropism in adult mice via tail vein delivery. Our pilot experiment showed that one rAAV5.NAEK mutant exhibited 2-fold higher lung transduction than rAAV5, whereas liver, heart, and skeletal muscle transduction was comparable. The enhanced lung tropism of rAAV5.NAEK was also observed following intranasal (IN) administration. Following IN delivery, immunostaining of lung sections and Western blot of tissue lysates revealed that the rAAV5.NAEK mutant showed lung transduction that was 4-folder higher than rAAV5, 12-fold higher than rAAV6, and 9-fold higher than rAAV6.2FF. Alveolar type II cells were the main targets of both rAAV5 and the NAEK mutant as demonstrated by immunofluorescence staining, attributing the enhanced transduction to higher transgene expression rather than altered cell type specificity. Furthermore, in vitro assays using A549 cells, a human lung epithelial cell line, corroborated the enhanced lung transduction in mice, and demonstrated that cell binding and internalization of the rAAV5.NAEK mutant were significantly more efficient than rAAV5. Finally, we performed structural modeling of the NAEK-modified AAV5 capsid. Interestingly, we found that the long side chain of NAEK possibly impacted on a neighboring VR near the three-fold axis, a core area of capsid-receptor interaction, indicating that a synergistic structural change involving multiple VRs may contribute to the enhanced lung tropism. Recent advances in genetic code expansion allow for translational incorporation of an increasing number of ncAAs that possess diverse biological properties. Our study

suggested that ncAA incorporation into AAV capsid may confer novel vector properties, opening a new and complementary avenue for rAAV discovery. (*Co-first authors; #Co-corresponding authors)

1158 AAV Promoters for Ectopic Gene Expression in Absence of Rep or Viral Helper Factors

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Adeno-associated virus type 2 (AAV2) has three promoters to control viral expression: p5, p19 and p40. Several studies suggest a mechanisms of transactivation of AAV promoters by Rep proteins. In the absence of helper Adenovirus (AdV), Rep cooperates with cellular proteins to inhibit AAV promoters' transcription, repressing its own synthesis, through p5. After AdV infection, Rep promotes a cascade of reactions that activates promoters p19 and p40, inducing the synthesis of Rep and capsid proteins, respectively. Presumably, this activation is dependent on Rep ability to form complexes with other proteins inducing a DNA structural distortion at AAV promoters proximate[1]. The potential and strength of each AAV promoter to drive the expression of ectopic genes in mammalian cells, in the absence of Rep or helper factors, is not completely known. This works aims at further elucidating it and pave the way for their manipulation. In this work, to study independent AAV promoter activity, we developed several constructs containing AAV2 promoter p5, p19 or p40, driving the expression of reporter gene, *eGFP* or *Gluc* (*Gaussia luciferase*). Two different promoter sequences were tested to detect the minimal active region. The mammalian 293 cells and non E1-coding region cell lines, such as A549 and Vero, were transiently transfected. Promoter strength was measured by flow cytometry (FC) for GFP or by luminometer for Gluc, and compared to a mock control. In contrast to previous reports[2], two days after transfection, we observe constitutive GFP expression in all cells evaluated and from all AAV promoters. Promoters p5 and p40 shown the highest sactivity. Promoter p19, was reproducibly the weakest promoter. These data, show that AAV promoters can be used to drive constitutive expression of non-AAV genes, with different strengths, independently of Rep, adenoviral genes, or cell origin (human or monkey). We have found that the addition of an intron region, downstream p40 promoter decreased gene expression in both tested cells. A minimal region of less than 160 bp, for promoter p5 and p40 presented similar strength to cellular promoter hPGK. Additionally, we observed that in the presence of adenoviral helper factors (i.e E2A, E4 and VARNA) AAV promoters and cellular hPGK promoter presented higher activity. AAV promoter conformations with inducible potential are being tested. These results provide insights on how to design optimal expression cassettes, that can be of major importance in the development of an AAV cell-based platform production systems, devoid of helper AdV. **References:** 1- doi: 10.1128/jvi.76.16.8225-8235.2002. / 2- doi: 10.1128/JVI.00387-0

1159 Preclinical Proof-of-Concept of AMT-260, a Novel AAV9-Dual microRNA-Based Vector Targeting GRIK2 for the Treatment of Temporal Lobe Epilepsy

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The objective of this study was to demonstrate that lowering of the expression of the Glutamate Ionotropic Receptor Kainate Type Subunit 2 (GluK2) using RNA interference against its mRNA GRIK2 can be a therapeutic strategy for temporal lobe epilepsy (TLE). TLE is the most common form of focal epilepsy characterized by recurrent seizures generated in the hippocampus. Patients with TLE are often resistant to anti-seizure medications. Mossy fiber sprouting from dentate granule cells (DGCs) is a consistent anatomical hallmark of TLE, leading to synaptic reorganization in an aberrant excitatory network. In TLE, the DGCs operate via aberrant ectopic expression of GluK2/GluK5 receptors and GluK2/GluK5 was demonstrated to play a central role in the generation of seizure activity. However, the pharmacological potential of GluK2/GluK5 as a target for the treatment of TLE remained to be tested. The work presented here reveals the preclinical efficacy and safety of an AAV9 based vector, AMT-260, to knockdown GluK2 expression level. AMT-260 is an AAV9-vector carrying two microRNAs under the control of a neuron-specific human synapsin 1 promotor targeting GRIK2. The vector was tested in the pilocarpine mouse model for TLE and using hippocampal organotypic slices from patients with TLE. Injection of increasing doses of AMT-260 in the hippocampus of mice treated with pilocarpine showed a dose-dependent decrease in the number of electrical seizures per day, reduced hyperlocomotion and improved health. A full biological effect was observed at 5.0E+09 gc per hippocampus in pilocarpine mice. Efficacy was also observed in organotypic slices from patients with TLE treated with AMT-260 with a decrease in the number of electrical seizures recorded. The safety and biodistribution of AMT-260 was assessed in exploratory and GLP NHP studies after 1 and 3 months of treatment following convection enhanced, MRI guided intrahippocampal delivery of AMT-260, similar to the route proposed for clinical administration. At doses ranging from 6E+10 to 1.2E+12 gc/hippocampus of AMT-260, vector DNA was concentrated in the hippocampus and adjacent entorhinal cortex but orders of magnitude less in other brain regions and no or negligible peripheral exposure. High neuronal expression of the microRNA in the hippocampus was associated with substantial knockdown of GRIK2 mRNA (up to 90%) and GluK2 protein. No adverse findings were reported in the NHP. With the preclinical efficacy in TLE and a good safety profile, AMT-260 will proceed into clinical testing for the treatment of patients with refractory mesial temporal lobe epilepsy.

1160 Data Compilation and Novel Methods for Convection-Enhanced Delivery Using a SmartFlow® Cannula for Gene Therapy Products to the Nervous System of Large Animals

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Accurate delivery of therapeutics into the central nervous system (CNS), brain and/or spinal cord is associated with a range of challenges. Implementation of imaging tools such as real-time magnetic resonance imaging (MRI) has led to the development of improved delivery protocols critical in preclinical and clinical studies. For this presentation, we have compiled data to demonstrate accuracy, effectiveness, and safety of delivering gene therapy vectors using the SmartFlow MR Neuro Ventricular Cannula. Data obtained from multiple studies conducted on more than N=50 nonhuman primates receiving intra-cranial infusions will be presented to demonstrate neurosurgical trajectory, positioning of the cannula within the selected anatomical structure, visualization/distribution/expression of the gene therapy product, histological analysis and post-surgical outcomes. In addition, as part of this presentation, novel data will be presented to showcase implementation and applicability of this technology in performing intra-spinal delivery, as well as intra-peripheral organ infusions using Omnipaque as the contrast agent. Approaches detailed herein will provide novel methods for the delivery of therapeutics within the brain, spinal cord, and peripheral organs, which are important considerations when targeting neurological disorders with a central and peripheral component.

1161 Synthetic Receptors Enable Regenerative Engineering for the Treatment of Osteoarthritis

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Introduction: Osteoarthritis (OA) is a joint disease that affects over 32 million Americans, leading to pain and limited mobility. Cartilage catabolism, which exposes a collagen II (CII)-rich extracellular matrix, is one biophysical hallmark of OA brought about by several factors including inflammation from cytokines such as IL-1. Treatment of the disease is either palliative or involves surgical intervention; there are no clinically approved disease-modifying OA drugs. Current applications of cell engineering strategies to treat OA rely on unregulated expression of therapeutic transgenes and thus do not govern behaviors based on OA-specific pathology. Here, we present a cell design strategy using the synthetic Notch (synNotch) receptor platform. SynNotch is based on native Notch signaling, which requires an immobilized ligand for receptor activation. By engineering a synNotch extracellular domain (ECD) to recognize exposed CII, as well as an intracellular domain (ICD) to regulate transgene expression, cells can recognize OA pathology and perform regenerative functions to ameliorate the arthritic environment. **Methods:** We licensed cells to recognize degraded cartilage by engineering an OA-specific synNotch ECD via an scFv for a monoclonal antibody targeting CII (mAbCII). A synthetic transcription factor served as the ICD (**Fig. 1A**). Lentiviral vectors encoding the receptor and inducible payload transgenes were used to transduce CII-synNotch mouse mesenchymal stromal cells (CII-synNotch MSCs) with gene circuit elements. For solid-phase CII experiments, CII was passively adsorbed to culture vessels overnight. For porcine cartilage experiments, explants were cryosectioned and seeded with CII-synNotch MSCs. Luminescence was measured with a luciferase assay. Protein concentrations were determined via ELISA. 0.1 ng/ml IL-1 was used to upregulate inflammatory gene expression of ATDC5 mouse chondrocytes. Gene expression was assessed via qRT-PCR. **Results:** CII-synNotch MSCs achieve robust activation on 25 µg/ml solid-phase CII (**Fig. 1B**). Engineered cells expressing inducible mCherry on matrix-exposed cartilage explants reveal spatial regulation of the gene circuit (**Fig. 1C**), proving that a mAbCII-derived synNotch receptor enables activation only in the context of an OA-specific biomarker. CII-synNotch MSCs also programmably secrete anti-inflammatory IL-1 receptor antagonist (IL-1Ra) and pro-anabolic TGFβ-3 **(Fig. 1D-E)**, achieving versatility in therapeutic function. To assess synNotch function in the context of inflammatory signaling, CII-synNotch MSCs engineered to inducibly express IL-1Ra were plated on CII and co-cultured with IL-1-dosed mouse chondrocytes. IL-1Ra-producing MSCs antagonized inflammation in a CII-dependent manner (**Fig. 1F**). Modulation of gene expression illustrates the utility of engineered cells at detecting and combating OA-associated pathology. **Conclusions:** Using CII as an OA biomarker for synNotch transgene expression enables cells to be functional detectors of arthropathies and serve as living therapies. The regulation conferred by CII-synNotch MSCs may circumvent off-target effects of biologic drugs by restricting expression to niches displaying direct signatures of OA pathology. This work demonstrates enhanced cell engineering for musculoskeletal therapies and can be extended to include expression of other biologic drugs that may aid in the mitigation of OA.

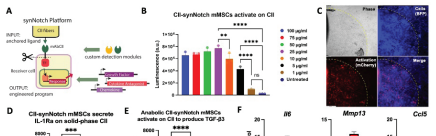

Figure 1: Development and validation of a CII-synNotch cell-based therapeutic for OA. **(A)** The synNotch platform works by (1) receptor recognition of exposed CII in damaged cartilage; (2) cleavage at the transmembrane core; (3) release of a transcription factor that binds to an inducible promoter and promotes transgene expression. **(B)** synNotch mMSCs respond on doses of CII as low as 5 µg/ml and most robustly at 25 µg/ml (t=72 hrs.) measured via inducible luciferase expression. **(C)** CII-synNotch cells (constitutive BFP) activate spatially restricted transgene expression (inducible mCherry) only when growing on matrix-exposed porcine cartilage tissue (t=5 days). Yellow dash: cryosection border. Scale = 200µm. **(D-E)** Anti-catabolic IL-1Ra and pro-anabolic TGFβ-3 are produced on solid-form CII but are undetectable in control conditions (t=72 hrs.). **(F)** Gene expression profiling of mouse chondrocytes reveals antagonization of inflammatory expression when co-cultured with activated IL-1Ra-secreting CII-*synNotch* mMSCs. *Statistical analysis: (*) p < 0.05; (**) p < 0.002, (***) p < 0.0002, (****) p < 0.0001.*

1162 Genome-Wide Analysis of Triple-Play Plasmid Integration in Pinnacle PCL™ Producer Cells Lines across Multiple Recombinant AAV Programs

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We have developed a producer cell line (PCL) manufacturing platform (Pinnacle PCL) for production of recombinant AAV (rAAV) gene therapy products, which has demonstrated scalable 2000L production in an industrial setting to support ongoing Phase 1/2 and planned Phase 3 clinical trials. PCL generation is initiated via transfection of a single "triple-play" plasmid (pTP) containing the AAV rep-cap genes, a rAAV

transgene cassette flanked by AAV ITRs and an antibiotic resistance gene, followed by application of selective pressure to isolate stably integrated cells. Stable integrants are isolated, single-cell seeded via Verified In-Situ Plate Seeding (VIPS) and monitored for monoclonality with cell imaging analysis during outgrowth. Top clones, and rAAV generated from these top clones, are analyzed for desired phenotypic and genotypic qualities via program-specific and program-agnostic assays. Here, utilizing targeted locus amplification (TLA), we present genome-wide analysis of pTP integration in Pinnacle PCL clones from 6 gene therapy programs utilizing 4 AAV serotype capsids. TLA selectively amplifies DNA, both genomic and episomal, that is spatially proximal and can be crosslinked, which is then coupled with Illumina short-read next-generation sequencing to detect integrated pTP sequences and mapped to the point of insertion into the HeLa producer cell line genome. Interestingly, most pTP integration occurs within chromosome 19, averaging ~66% for all generated clones. Given the significantly higher level of integration at chromosome 19 than other genomic regions, especially when compared to predicted rates of random integration, we speculate that transient, low-level Rep 78/68 expression from the pTP during transfection is sufficient to drive this phenomenon. Importantly, integrated genome copy (IGC) assays were completed with 3 separate pTP ddPCR targets to verify the integration and copy number of pTP integrants in these Pinnacle PCL clones. Finally, a critical aspect of Pinnacle PCL development and characterization is the completion of a 16-week stability study, which is designed to determine if top clones demonstrate the phenotypic and genotypic stability required for 2000L manufacturing campaigns. TLA analysis of monoclonal PCLs generated for 4 gene therapy programs harvested at early and late time points in the 16-week stability study demonstrated genotypic, as well as phenotypic stability.

1163 Adeno-Associated Virus (AAV) Loaded Hydrogel Implants Improve GFP Expression and Reduce Anti-Drug Antibody Titers (ADA) and Ocular Inflammation in Rabbits

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Purpose: Intravitreal (IVT) dosing of AAVs often induces an inflammatory response that increases with dose. In this study, we tested how the pharmacokinetics of AAV delivery via a degradable hydrogel implant can impact ocular inflammation, immune response, and transgene expression. **Methods:** We prepared AAV2.7m8-CMV-GFP loaded hydrogel implants at a dose of 3.6E+10 GC/implant to release AAVs over 4 (Fast Release) or 14 days (Medium Release). A single implant was injected intravitreally in both eyes of New Zealand White rabbits. Bilateral injections of a bolus 50µL AAV solution at the same dose were performed as a positive control. Inflammation in rabbit eyes was assessed via ocular examinations (n=3 rabbits/6 eyes per group). Green fluorescent protein (GFP) expression was evaluated through fundus autofluorescence (FAF) imaging (n=3 rabbits/6 eyes per group) and enzyme-linked immunoassay (ELISA) quantification in ocular tissues (n=3 rabbits/3 eyes per group). Vector copies in rabbit plasma were detected by quantitative polymerase chain reaction (qPCR) and ADA titers in serum were determined by ELISA. **Results:** The greatest amount of GFP was expressed in rabbit ocular tissues with the medium release AAV group (Figure 1). Ocular examinations and ELISA test results showed the Medium release group had lower peak inflammation at Week 3 and lower ADA titer levels at Week 13 compared to the other groups (Figure 2A & 2B). Modulating release kinetics of AAVs via hydrogel implants can also reduce systemic levels of AAV as evidenced by lower copy numbers from the AAV implant groups in plasma (Figure 2C). **Conclusions:** In this study, we show that instead of bolus injection, delivering a high dose of adeno-associated virus (AAV) over time using a sustained-release modality could reduce inflammation, lower ADA titers in serum, and improve transgene protein expression. These data demonstrate that controlled release AAV implants may provide a therapeutic benefit for ocular gene therapy.

Figure 1. Quantification of GFP in rabbit ocular tissues via ELISA and representative Week 12 FAF images.

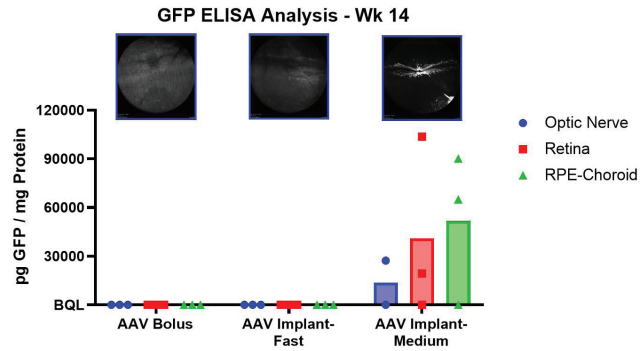
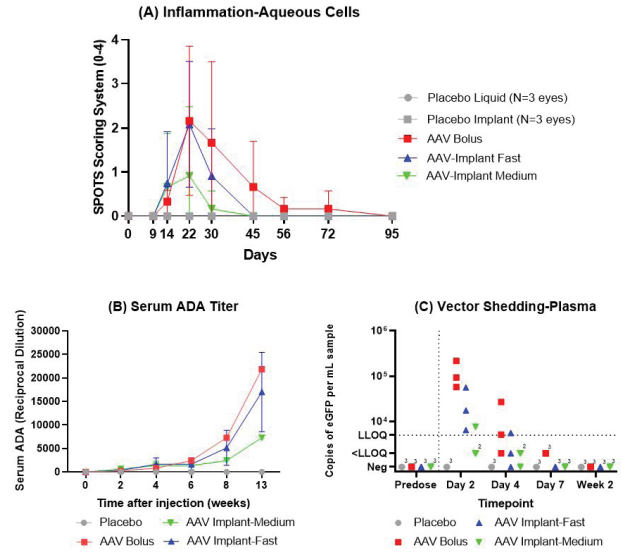


Figure 2. Effect of controlled release of AAVs on (A) inflammation-aqueous cells, (B) ADA titers, and (C) vector shedding.



1164 A Functionally Enhanced Tumor-Infiltrating Lymphocytes Product (GT201) Exhibits Improved Persistence and Anti-Tumor Efficacy with Low IL-2 Dependency

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Adoptive cell therapy by tumor-infiltrating lymphocytes (TIL) has demonstrated promising clinical benefits for patients with late-stage solid tumors. However, high-dose IL-2 used as standard adjuvant for TIL therapy requires close clinical monitoring and may cause toxicities. Furthermore, the prone-to-exhaustion status of final TIL product after rapid expansion in vitro as well as the immunosuppressive tumor microenvironment further limits the persistence and efficacy of TIL post infusion into patients. Grit Biotechnology designed a genetically engineered TIL product (GT201) to express a membrane-bound cytokine that is critical for T cell survival and function. Different liker designs of GT201 were examined and the best design was selected based on stable expression of GT201 construct on T cell surface and increased cis and trans signaling, therefore enhanced functional impact on TIL. GT201 showed significantly improved long-term survival and preservation of TCR clonality without IL-2 in vitro, as well as persisted longer in multiple tissues without IL-2 than conventional TIL + IL-2 in NOG mice. In the meanwhile, anti-tumor function was also significantly enhanced in GT201 as demonstrated by increased cytotoxicity and cytokine production against tumor cells in vitro and better tumor control ability during serial killing assays. Notably, GT201 TIL achieved better anti-tumor efficacy with low IL-2 than conventional TIL with normal IL-2 in a PDX mouse model treated with autologous TIL, corelated with higher IFN-γ release in mouse serum. Further mechanistic studies demonstrated that GT201 mainly act through cis signaling, but GT201 transduced TIL would not survive over two months without IL-2 in culture. If working by trans signaling, it would preferentially modulate activated T cells and NK cells, for instance, tumor reactive immune subsets in the cancer setting, rather than their naive counterparts, suggesting a low possibility of over-activation of immunity. Taken together, GT201 demonstrated enhanced persistence and anti-tumor efficacy with low IL-2 dependency in vitro and in vivo. Currently, manufacturing process of GT201 TIL product has been successfully developed (StaViral®), and clinical assessment of GT201 as a next-generation TIL therapy is ongoing in an investigator-initiated trial in China.

1165 Discovery of Novel AAV Capsids with Enhanced Skeletal Muscle Tropism Following Directed Evolution in Cynomolgus Macaque

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The application of recombinant AAV vectors for therapeutic gene delivery holds substantial promise in the treatment of musculoskeletal and neuromuscular disorders. However, sufficient muscle targeting by existing AAV vectors in humans necessitates high systemic doses, which have been associated with adverse events across several clinical trials. Thus, there is a need to identify novel vectors with enhanced muscle targeting, which may improve gene delivery efficiency and allow lower doses to be used. Here, we report the discovery of a set of skeletal muscle-tropic AAV capsid variants in cynomolgus macaque. Prior engineering efforts have suggested that incorporation of an integrin-binding Arg-Gly-Asp (RGD) motif on the AAV capsid surface may enable redirection of vectors to integrin heterodimers. To investigate whether RGD motif-displaying AAV capsids can be effectively retargeted, we inserted a 7-mer peptide containing an embedded RGD motif in variable region VIII (VR-VIII). To construct the plasmid library, capsid-coding sequences were placed between the AAV inverted terminal repeats (ITRs) so that each produced capsid would package its own originating genome. This AAV library was iteratively screened via intravenous administration in cynomolgus macaques, and skeletal muscle-recovered capsid cDNA was cloned back into the library backbone for subsequent rounds of screening. In parallel, we monitored capsid variant abundance at each screening stage of directed evolution through short-read sequencing. We observed significant enhancement in skeletal muscle transduction by Round 2 compared to Round 1 AAV library, suggesting positive selection on muscle-targeting variants. From the sequencing analysis, we identified a set of RGD motif-displaying capsids with enhanced transduction in skeletal muscle, with top variants demonstrating over 20-fold enhancement compared to benchmark. Further sequence analysis uncovered distinct peptide motifs that exhibited consistent enrichment. Additional comparative analysis on liver and DRG transduction confirmed that enrichment of these novel variants was generally more specific to skeletal muscle. Presently, further characterization of these capsids is in progress, and validation studies are underway.

1166 Production of AAV Vectors in a Scale-X™ Fixed-Bed Bioreactor

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The adeno-associated viral (AAV) vector has emerged as a viable option for many gene therapy applications due to its low immunogenicity, broad tissue specificity, and long-term transgene expression. To meet the growing demand for large quantities of AAV required for clinical trials and treatment, a scalable production technology of AAV vectors

is needed. Fixed-bed bioreactors offer an advantage in the production of AAV vectors by providing a large-scale and controllable platform equally suitable for both adherent and suspension cells. scale-X™, a commercialized fixed-bed bioreactor from Univercells Technologies, has a large surface area based on a spiral-wound double-layer structure similar to 2D cell culture environment. Therefore, we aimed to develop large-scale AAV production using the scale-X hydro bioreactor. As a first approach, we applied the production parameters from our lab-scale production system. The viral productivity of our first bioreactor run did not reach our expectations due to low transfection efficiency and cell death caused by the toxicity of calcium phosphate. We then developed a polyethylenimine (PEI)-based transfection method, focusing on the DNA amount and PEI:DNA ratio. We also added a rinsing technique after transfection to reduce residual transfection agent effects. The scale-X hydro bioreactor, with a 2.4m^2 surface area, showed similar viral productivity per area as the 2D lab-scale system. We also compared the infectivity of AAV produced by the bioreactor and cell culture plates. Flow cytometry results demonstrated that the scale-X bioreactor could produce AAV vectors with high infectivity compared to the 2D lab-scale system. In addition, The scale-X nitro (600m^2), a further compatible scalable option provided by Univercells Technologies, will meet the requirements for large-scale clinical trials.

1167 Chunk Editing: A Novel Method to Extend the HDR "Rewriting" Window from 10 to 1000s of Base Pairs

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CRISPR enabled cell and gene therapies have the potential to revolutionize the field of genetic medicine. However, the vast majority of rare diseases remain untreatable due to the limitations of current tools and techniques. To date, most non knockout (KO) based therapeutics have been restricted to mutation-by-mutation approaches, where either HDR, or newer techniques (base/prime editors), rewrite small regions of DNA at a time (~1-100 bp). While these approaches are powerful, short editing windows (relative to the size of human genes) are financially and/or technically incompatible with most rare-disease mutation profiles. Here, we demonstrate for the first time that CRISPR/Cas9 can be used to "rewrite" 4kb+ sections of the human genome simultaneously via a selection-free process we have named "chunk editing". In these experiments, H1299 and HEK293 cells were transfected with plasmids that encoded guides, nuclease, and DNA donor repair template using lipofectamine 3000. Three days post transfection, cells were lysed and genomic DNA was extracted, amplified, and prepared for sequencing on either Illumina MiSeq or NextSeq sequencers. Standard tools were used to filter and align the resulting sequencing data which was then quantified via custom code. Our first target was exon 14 of Factor 8, a therapeutically relevant target with over 65 individual mutations classified as likely-pathogenic or pathogenic in NCBI's ClinVar database. H1299 cells were transfected as above, with the repair template containing 12 introduced SNPs scattered throughout the ~3.3kb editing window. Using our novel chunk editing techniques, we achieved remarkably consistent editing rates (~2-3% of total reads sequenced) across the entire editing window of exon 14 (Fig 1A, blue line) with two controls (transfected without guide and untransfected; dashed blue and black lines respectively) showing minimal background levels of editing (<0.1%). To determine whether this chunk editing strategy is necessary to allow for long-range editing, we assayed another locus, exons 6-8 of the PAH gene, introducing 7 SNPs over an almost ~4kb editing window. As expected, traditional HDR approaches (purple) showed a marked drop in efficiency outside of the expected ~10bp window (from 0.6% to <0.1%), whereas chunk editing (blue) showed a much more even editing profile (0.4-0.6%) throughout the whole editing window. Chunk editing in HEK293 cells showed remarkably similar results (0.6-0.8%), demonstrating that this technique can be used in multiple cell types and genomic loci. While future work remains to explore and optimize chunk editing strategies, we believe the ability to extend HDR based editing windows to thousands of base-pairs will have a tremendous impact on future therapeutic and R&D applications.

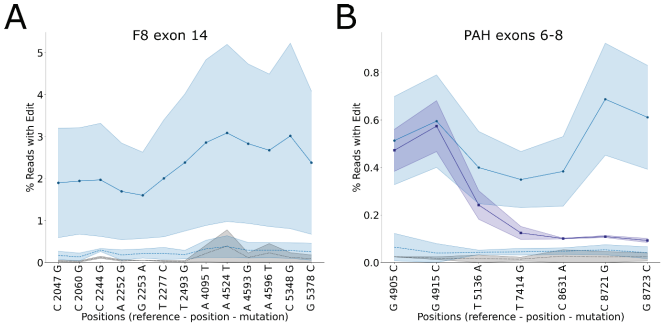

Figure 1 Legend: **1A**: Graph showing editing rates across 3kb+ of genomic DNA using chunk editing strategy. nuclease, guides, and repair template (RT) in blue; nuclease and RT (no guides) dashed blue; untransfected control in dashed, black. Shading shows the Standard Error of the Mean (SEM) for each treatment, n=3 per treatment. **1B**: Graph showing editing rate for legacy approach (purple) or chunk approach (light blue, solid line), with controls pattern as in 1A. Shading shows the Standard Error of the Mean (SEM) for each treatment, n=3 per treatment.

1168 Self-Adjuvating Lipid Nanoparticle mRNA Vaccine Elicits T-Cell Responses and Protects Against Immune Challenge

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BACKGROUND: Nucleic acid vaccines, DNA and messenger RNA (mRNA), have emerged as promising modalities for infectious disease and for cancer immunotherapy due, in part, to shortened manufacturing cycles and high potency. Currently, there is limited understanding of the mechanisms of antigen presentation and induction of specific T-Cell responses critical to long-term immunity. Lipid nanoparticles (LNP) composed of ionizable lipids are important components of such vaccines as they can convey and present the nucleic acid effectively to the immune system. Further improvements in LNP carriers require a reduction in the 1) systemic toxicity, 2) improved endosomal escape, 3) potent and T-cell specific adjuvating function and 4) targeting to specific antigen presenting cells. RESULTS: Previously we have reported that lipid nanoparticles

composed of a novel ionizable, biodegradable lipid ("SS-Lipid") can deliver pDNA or mRNA in mice to liver, solid tumors, and other organs via the IV route and achieve high levels of expression. We also evaluated the safety of the lipids in mice where total lipid doses of up to 175 mg/kg were well tolerated. We next evaluated the delivery of DNA and RNA vaccines via the SC route. Multiple SS-Lipid derivatives were synthesized and identified one lipid (SS-EC) that specifically activated macrophage cell in vitro. Antibody generation response was assessed in BALB/c mice with SS-EC 1.5x μg OVA mRNA injected 3X at weekly intervals and compared with immunization with OVA protein+polyI:C. SS-EC LNP produced a 2.2X higher titer compared with OVA-polyI:C control (Figure 1A). To assess induction of cytotoxic t-cell activity, SS-EC-LNP carrying OVA-encoding mRNA or Luciferase-encoding mRNA were administered at a dose of 0.05 μg of mRNA. The CTL percent lysis activity at 1 week was 75% (SS-EC_OVA-mRNA) of pulsed antigen cells. Sufficient CTL activity was not obtained with other DOTAP liposome, SS-EC_Luc-mRNA and empty LNP (Figure 1B). We then evaluated if robust CTL responses could demonstrate activity against solid tumors. Mice were injected with OVA expressing E.G7 cells as a model for disseminated malignant disease, and treated with SS-EC LNP OVA-mRNA (0.2 μg) after 10 days or SS-EC Luc mRNA as negative control. Tumor growth was significantly repressed in the test animals with negligible growth by day 25 (Figure 2). Additional data on the development of a room temperature stable formulation of SS-Lipid mRNA drug producct will be presented. CONCLUSION: We here demonstrate that a novel ionizable lipid SS-E and SS-EC specifically activates macrophages in vivo and this response is correlated with antibody expression and Cytotoxic T-Cell activity. T-Cell responses were shown to be effective in limiting growth of tumor cells in a disseminated disease model. Further efforts are needed to optimize the dosage and formulation towards achieving a single dose vaccine product. These findings suggest LNP composed of SS-E and SS-EC can be effective delivery systems for DNA and RNA vaccines.

FIGURE 1:

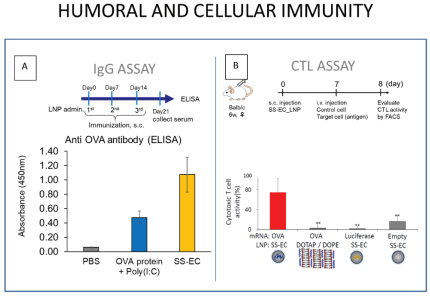
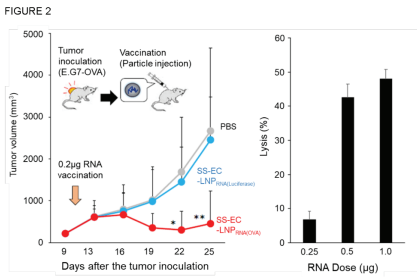


FIGURE 2



1169 Optimization of a Versatile Downstream Process for Multiple Novel AAV Capsids That Demonstrates Improved Recovery

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The gene therapy field has seen a surge in treatment candidates using recombinant adeno-associated viruses (rAAVs). The field is exploring novel capsids, derived from wildtype serotypes, to achieve more efficient gene delivery. While novel capsids are more efficient delivery vehicles, they may perform unexpectedly in purification platforms established with wildtype serotypes. Additionally, novel capsids may yield higher titers and have characteristics (e.g., charge, percent full, etc.) impacting compatibility with pre-defined buffers and the sizing of process components. Adapting downstream steps is crucial for the successful development of a manufacturing-scale process. Through a series of screening experiments at different scales with two commercially available resins and a range of buffer formulations, a toolkit of buffers specific to AAV affinity-based chromatography was developed. Affinity step recovery for multiple novel capsids was improved 30-50% through this optimization process. Recoveries in line with wildtype serotypes were achieved. In addition to optimization of the affinity capture step, recovery was improved by 20% and 60% through optimization of ultrafiltration parameters and filtration sizing, respectively. These optimizations were performed at bench scale and subsequently confirmed at larger development scales. Through process optimization and parameter screening a robust purification process was developed for multiple novel capsids.

1170 AAV-Mediated Gene Augmentation Therapy of *CRB1* Patient-Derived Retinal Organoids Restores the Histological and Transcriptional Retinal Phenotype

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Background: Mutations in both alleles of the Crumbs homologue 1 (*CRB1*) gene mostly cause retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA), whereas some mutations cause macular dystrophy. CRB1 and CRB2 are family members and large transmembrane proteins consisting of a short 37 amino acid intracellular domain containing a conserved C-terminal glutamic acid-arginine-leucine-isoleucine (ERLI) PDZ binding motif and a protein 4.1, ezrin, radixin, moesin (FERM) motif juxtaposed to a single transmembrane domain, and a large extracellular domain with multiple laminin-A globular and epidermal growth factor (EGF) like domains. CRB1 and CRB2 proteins are required for organizing apical-basal polarity and adhesion between photoreceptors and Müller glial cells. CRB1 and CRB2 localize at the retinal outer limiting membrane at a subapical region adjacent to adherens junctions between Müller glial cells and photoreceptors. So far, no treatment possibilities are available for patients with RP or LCA caused by mutations in the *CRB1* gene. Previous studies in *Crb1* and *Crb2* knockout mice showed proof of concept for AAV2/9.CMV.h*CRB2*, but not AAV2/9.CMVmin.h*CRB1*,

transduced onto Müller glial cells and photoreceptors. Transduction of only Müller glial cells or only photoreceptors was not effective, whereas transduction of both cell types was. Here, we studied the effects of 4.8 kb AAV2/5.CMV.h*CRB2* and 4.8 kb AAV2/5.CMVmin.h*CRB1* gene augmentation vectors on multiple independent biological replicates of *CRB1* patient retinal organoids with c.3122T>C p.(Met1041Thr) homozygote missense mutations, or with 2983G>T p.(Glu995∗) and c.1892A>G, p.(Tyr631Cys) compound heterozygote mutations, or with c.2843G>A p.(Cys948Tyr) and c.3122T>C p.(Met1041Thr) compound heterozygote missense mutations. **Results:** *CRB1* patient-derived induced pluripotent stem cells were differentiated into *CRB1* retinal organoids. Surprisingly, the *CRB1* retinal organoids with missense mutations show a near complete loss of variant CRB1 protein at the outer limiting membrane by immunohistochemical analysis. Interestingly, the localization of the CRB2 protein or other CRB-interacting protein is not affected. Compared to isogenic controls, the *CRB1* patient organoids show moderate but significant loss of photoreceptors at differentiation day 210. Single cell RNA-sequencing (scRNAseq) of the *CRB1* patient and isogenic controls showed clustering of cells into Müller glial cells, stromal cells, mature rods, immature rods, cones, transient cell populations, bipolar cells, retinal pigment epithelial cells, proliferating cells, amacrine cells, astrocytes, horizontal cells and ganglion cells. ScRNAseq at differentiation day 230 revealed changes in the endosomal pathway and cell adhesion and migration in *CRB1* patient-derived retinal organoids compared to their corresponding isogenic controls. Adeno-associated viral serotype 5 (AAV2/5).CMV.*GFP* efficiently transduced Müller glial cells in addition to photoreceptors and retinal pigment epithelium at differentiation day 120, whereas (AAV2/2).CMV.GFP efficiently transduced photoreceptors and retinal pigment epithelium at differentiation day 120. AAV2/5.CMV.h*CRB2* or AAV2/5.CMVmin.h*CRB1* gene augmentation in Müller glial and photoreceptor cells partially restored the histological phenotype and transcriptomic profile of *CRB1* patient-derived retinal organoids as determined by scRNAseq. **Summary:** Altogether, we show proof-of-concept that AAV.h*CRB1* or AAV.h*CRB2* treatment improved the phenotype of cultured *CRB1* patient-derived retinal organoids, providing essential information for future gene therapy approaches for patients with mutations in the *CRB1* gene.

1171 Optimizing Adeno-Associated Virus-Based Gene Therapy for the Treatment of Surfactant Protein B Deficiency

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Background: Surfactant protein B (SP-B) deficiency is an inherited disorder that leads to respiratory failure immediately after full-term birth. With the limited treatment options offering only transient improvement, this disease is lethal within the first year of life. Given that SP-B deficiency only affects the lungs, targeted delivery of gene therapy to the respiratory tract holds promise in treating this disease. With the proliferation of gene therapy clinical trials, safety and serious adverse events emerge as key considerations for drug product development. Many AAV gene therapies continue to use highly active promoters to drive the expression of therapeutic transgenes. Although these ubiquitous promoters are well-established and confer strong transgene expression, they continue to present disadvantages. A common problem observed is non-targeted transgene expression that can result in compromised cell health, inflammation, and toxicity. Our approach to address these safety concerns involves computational mining and engineering of lung specific promoters to drive gene expression in target tissue and prevent expression in off-target tissues that may cause adverse events or increase immunogenicity of the vector. Using our engineered AAV6.2FF vector expressing an alkaline phosphatase (AP) reporter gene under the control of 4 different putative lung specific promoters identified through computational screens, we evaluate targeted transgene expression in mice. **Methods:** AAV vectors were administered to C57BL/6 mice (n=4), with two additional control groups, one receiving 1xPBS as a negative control and the other receiving an AAV6.2FF vector expressing AP from the ubiquitous promoter CASI as a positive control. Mice were transduced with $1x10^{11}$ vector genomes (vg) intranasally, intramuscularly, and intraperitoneally to ensure targeting of all possible tissues. 21 days post-administration, mice were euthanized, and all major organs harvested and stained for AP reporter gene expression. Gross images of the tissues were taken, and AP activity assays were conducted to quantify the amount of AP protein per tissue. Histologic analysis will be performed to identify the various cell types where the promoters are active. **Results:** All but one of the promoters show efficient gene expression in the lung with minimal off target expression. AP assays confirmed low to undetectable AP expression in the brain, heart, liver, nasal cavity, pancreas, spleen, and trachea for all lung specific vectors. The positive control demonstrated strong transgene expression in many tissues outside of the lungs including the heart, liver, spleen, and muscle. Visually, lung transduction in the positive control and in 3 of the lung specific promoters appear to be very similar in magnitude (Fig. 1). **Conclusions:** The use of AAV6.2FF in conjunction with computationally predicted lung specific promoters can efficiently transduce the lungs while minimizing off target effects in the rest of the body. In addition to localizing transgene expression, lung specific promoters have the capacity to express just as effectively as well-established ubiquitous promoters thereby increasing the safety of AAV gene therapies for monogenic lung diseases like SPB-deficiency.

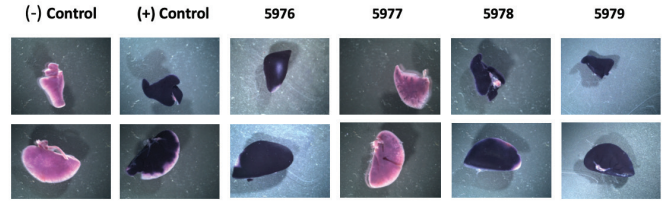

Fig 1. Human placental alkaline phosphatase expression in the lungs of C57BL/6 mice 21 days post AAV administration with various promoters identified through computational analysis.

1172 IsoTag®LV - A Scalable, Chromatography-Free Solution to Lentiviral Purification Using a Novel Affinity-Based Platform

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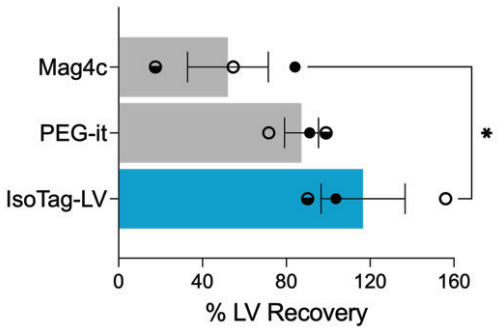
Isolere Bio, Durham, NC

The rapidly growing cell and gene therapy field has the potential to target a wide range of previously incurable diseases and cancers, due in no small part to the use of lentiviral vectors (LVs). LVs can target a variety of dividing and non-dividing cell types and can carry large genetic loads predominantly used for ex vivo transgene delivery. However, the fragility of LV envelopes, a critical component to their high potency, makes manufacturing difficult at each step of the production workflow. Anion exchange chromatography (AEX) and the many, complex downstream steps are the main contributors to low LV recovery due to long processing times and the requirement of harsh buffers. This leads to expanding upstream production volume, exponentially increasing the demand for expensive raw materials. These factors are a major reason why the costs of curative cell and gene therapies on the market and in clinical trials are so high. We developed a chromatography-free purification platform using our novel affinity reagent, IsoTag®LV, to streamline downstream purification into a two-step TFF process. The first step, a concentrate and wash of the crude harvest material, has a step recovery of over 90% and provides a two-log reduction of host cell proteins (HCP). The second step removes the IsoTag®LV reagent, providing highly pure LV with a step recovery of 60%. The entire process concentrates and purifies LV by 200X with a total functional recovery of over 50%. Improved recoveries are attributed to the use of gentle buffers as well as the inherent virus-stabilizing capabilities of IsoTag®LV. When mixed with LV, IsoTag®LV enhances viral stability to improve recoveries by over 50% in high salt, elevated temperature, and for an extended time at 4-8 °C. Furthermore, IsoTag™LV improved vector stability during freeze-thaw cycling and improved transduction efficiencies in T cells by 5x. The IsoTag®LV system is a revolutionary leap forward in LV downstream purification and successful implementation of this platform could significantly reduce the volumetric burden placed on lentiviral manufacturers and the cost burden placed on patients.



1173 AAV Capsid ELISA Automation for High-Throughput AAV Titer Determination

Dana Holzinger, Christina Sonnek, Katharina Hammer, Caroline Odenwald, Katja Betts
PROGEN Biotechnik GmbH, Heidelberg, Germany

Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. This development is reflected by the great influx of big pharma and new technologies into the field that could be observed over the last years. The fast growing environment allows a quick development due to the extensive investment; however, it also poses many challenges that need to be addressed. Among those is the competitive go-to-market time pressure as well as increasing numbers of samples that need to be processed and analyzed within the same time span. Consequently, there is a growing demand for more time-efficient solutions with less hands-on time for analytical characterization of AAV samples. Amongst the commercially available analytical tools, the AAV capsid ELISA is one of the most reliable and established methods for AAV total capsid titer quantification. To show that the AAV capsid ELISA is suitable for high-throughput processing, PROGEN has conducted a study. By transferring our established manual PROGEN AAV ELISA to a fully automated system, we have been able to show the potential for automation and high-throughput processing. This involved analyzing and validating the key performance indicators of our available AAV9 and AAV8 ELISAs (i.e. PRAAV9, PRAAV9XP, PRAAV8 and PRAAV8XP) using the fully automated system DSX from Dynex Technologies. The Dynex DSX is a fully automated 4-plate ELISA processing system, capable of performing several assays per plate simultaneously. This provides optimized efficiency and speed. Here, we show the data on homogeneity, intra- and inter-assay variance, as well as recovery obtained by the automated system compared to the same key performance indicators obtained by manual processing of the assays. The fully automated system has significantly reduced hands-on time, as well as higher sample throughput, whilst maintaining the benefits of the conventional AAV ELISA, i.e. robustness and accuracy in AAV titer determination. In conclusion, with this data we provide a high-throughput protocol for our AAV ELISA users who are analyzing high sample numbers per run and need to save time.

1174 Optimal Design of an AAV Vector is Critical to Evade Toxicity of Gene Therapy in a Mouse Model of Neuronopathic Gaucher Disease

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Gaucher Disease (nGD) is an inherited metabolic disorder caused by mutations in the *GBA1* gene. It is the most common lysosomal storage disease and can manifest with severe neurodegeneration and visceral pathology. The most acute neuronopathic form (type 2, nGD), for which there are no curative therapeutic options, is characterised by devastating neuropathology and death during infancy. In this study, we investigated the therapeutic benefit of systemically delivered AAV9 vectors expressing the human *GBA1* gene at two different doses comparing a neuronal-selective promoter with the

ubiquitous chicken-β-actin promoter, on survival, neurodegeneration, motor function, biochemical markers, and visceral pathology. High dose gene therapy resulted in extended life span of knock-out mice, normalisation of neuropathological markers, behavioural indexes, and increased enzymatic activity in brain and visceral organs. While supraphysiological expression of glucosylcerebrosidase (GCase) was beneficial in ameliorating the neuropathology, signs of inflammation were present in the viscera of mice treated with the ubiquitous vectors, suggesting that elevated GCase expression may promote inflammation and have deleterious effects in the liver and other visceral organs.

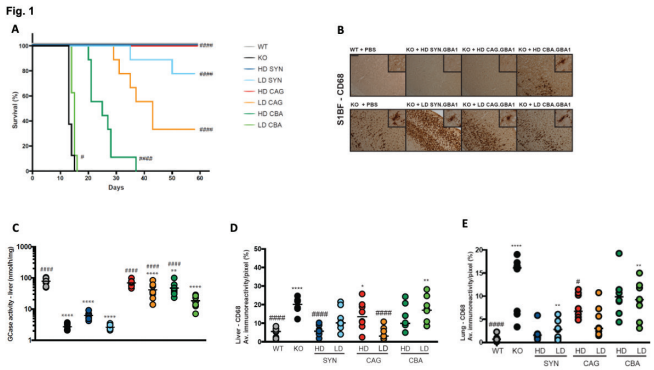

FIG. 1 Gene therapy rescues the K14-lnl/lnl model in a dose-dependent fashion and ameliorates neuropathology, while treatment with the ubiquitous vectors leads to increased inflammation in the viscera. ***A*** *Kaplan-Meier survival curve. Data expressed as percentage of survival. HD: high dose; LD: low dose.* ***B*** *Representative images of brain sections (cortical region S1BF) stained for the macrophagic marker CD68.* ***C*** *GCase enzymatic activity in liver homogenates.* ***D-E*** *Quantification of immunoreactivity of liver and lung sections stained for CD68. * indicates statistically significant difference between the experimental group and WT controls; # indicates statistically significant difference between the experimental group and untreated KO controls.*

We further evaluated the effect of sustained high-levels expression of GCase driven by the CAG promoter in a 1-year long-term study. Our long-term study showed that sustained GCase expression does not fully reverse the brain pathology, as demonstrated by the increased hyperactivity in the 1-year-old mice treated with CAG.hGBA. Our results highlight the importance of a careful evaluation of the promoter sequence used in gene delivery vectors, suggesting a neuron-targeted therapy leading to high levels of enzymatic activity in the brain but lower GCase expression in the viscera, might be the optimal therapeutic strategy for nGD.

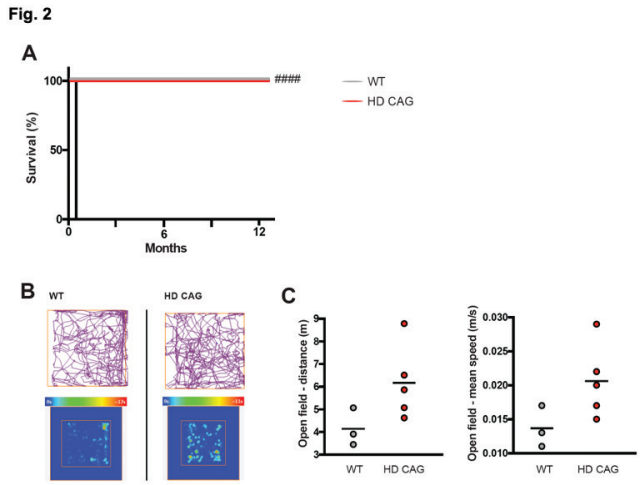

FIG. 2 Long-term study on mice treated with HD CAG.hGBA. ***A*** *Kaplan-Meier survival curve. Data expressed as percentage of survival. # indicates statistically significant difference between the experimental group and untreated KO controls.* ***B*** *Examples of open field traces and heat maps (total time spent in a specific position of the chamber during movement).* ***C*** *Distance travelled and mean speed travelled in the open field chamber in a 5-minute time period.*

1175 Development of SKG0106 Vector for the Effective, Safe and Durable Treatment of Neovascular Age-Related Macular Degeneration

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Anti-VEGF therapies revolutionized the treatment of neovascular age-related macular degeneration (nAMD), yet real world evidence suggest that long-term outcomes decrease over time and treatment complication occurs due to the burden of frequent intravitreal (IVT) injection. Therefore, a long-lasting and effective treatment is badly needed by patients with the condition. SKG0106 is a novel recombinant AAV vector we developed for the treatment of nAMD via IVT administration. SKG0106 comprises a proprietary capsid and a transgene genome that encodes an anti-VEGF protein, Nb24. Upon delivery, SKG0106 transduces retinal cells, and the expressed Nb24 effectively and specifically suppresses the action of human VEGF, a key factor contributing to the pathological angiogenesis of nAMD in patients. A number of pharmacological studies were conducted to thoroughly evaluate the efficacy of SKG0106 using a DL-α-AAA induced rabbit model of chronic retinal neovascularization (RNV) and a laser photocoagulation induced monkey model of choroidal neovascularization (CNV). In all studies, SKG0106 was administered via a single IVT injection. At pre-determined time points, the efficacy of SKG0106 was quantified in the rabbit RNV model based on the improvement rate of fluorescein leakage area and evaluated in the monkey CNV model based on 1) grade 4 laser spot rate, 2) mean

fluorescein leakage area, and 3) thickness of subretinal hyperreflective material (SHRM). The local tolerance in eyes was assessed by slit lamp microscopy. In the DRF study with rabbit CNV model, multiple doses of SKG0106 were administered, and the study lasted for 6 weeks. The treatment at all dosage levels significantly reduced fluorescein leakage area and exhibited a sigmoidal dose-response curve. The vector from the dose of 1E9 vg/eye and above completely inhibited fluorescein leakage, comparable to that of Eylea®. In addition, SKG0106 inhibited fluorescein leakage until the end of the study, while Eylea® lost its activity. The results suggested that SKG0106 was effective and lasting in repressing the activity of VEGF. Besides, the vector was well tolerated. In the DRF study with monkey CNV model, 6 doses of SKG0106 were administered (4-8 eyes/group), and the study lasted for 6 weeks. SKG0106 exhibited a dose-dependent effect in reducing CNV grade 4 spot rate and mean spot leakage area at 1-2 weeks post-modeling, the thickness of SHRM among the groups was also significantly reduced. The efficacy of SKG0106 at and above 8E9 vg/eye was comparable to that of Eylea®, and the vector demonstrated excellent tolerance profiles. In the prophylactic long-term CNV monkey study, animals were dosed with SKG0106 (6E10 vg/eye) for different observation periods of 6, 13 or 26 weeks. SKG0106 exhibited comparable efficacy to that of Eylea® at 6 and 13 weeks following administration. Importantly, although the efficacy of SKG0106 did not change in the 26-weeks groups, Eylea® did not show the inhibitory effect on the lesions. The thickness of SHRM in SKG0106 group was significantly thinner than that of vehicle group and Eylea® group. In addition, there was no adverse effect detected in the study. No systemic abnormal findings were detected in all pharmacological studies. SKG0106 in all preclinical studies exhibited strong dose-dependent efficacy against vascular leakage in rabbit RNV and monkey CNV models, durable prophylactic efficacy in monkey CNV model, excellent local tolerance profiles and systemic safety. With the support from all these data and the data from long-term GLP toxicity study (data not shown), the vector has entered into clinical stage. (Note: The binding affinity, blockade potency, biodistribution and pharmacokinetics of SKG0106 were studied and discussed in a separate abstract.)

1176 Advancing the High Density Cell Respirator Bioreactor (HDCR) to Overcome Bottlenecks Facing Cell and Gene Therapy

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Intensification of bioproduction processes will be key to overcoming the bottlenecks facing next-generation cell and gene therapy products. Towards this end, we present advancements to the HDCR bioreactor system, which has been engineered to provide a scalable, universal, and accessible platform for CGT products. The system leverages membranous gas exchange, micro-engineered cell expansion niches, and gentle media perfusion to support cell growth to 10^8 cell/mL densities in a virtually shear-free environment. The HDCR achieves a $k_La > 60$/hr and supports >100-fold expansion of producer cell lines (HEK, Vero, CHO, HeLa), which minimizes seed train demand. In contrast to fiber-based fixed bed bioreactors that tend to act like depth filters, cells are easily recovered intact from the bioreactor, extending application to cell-based products such as stem cells, NK, or CAR-T. Protocols have been established for adherent, suspension, and microcarrier-assisted cultures. The HDCR system comprises a "unit cell" architecture that can be linearly scaled in x and y, and stacked in z affording predictable, linear scaling. For AAV and oncolytic vector processes, we have demonstrated 6,000 fold scaleup while maintaining per-cell productivity. Upcoming studies in our new HDCR Midi platform aim to push scaleup 10-fold further. With an aim to improve operational efficiency in CGT CDMOs and process consistency, we have integrated a 21CFR Part 11 compliant control system, complete with real-time metabolite monitoring (glucose, glutamine, glutamate, lactate) as well as pH, DO, and temperature sensing. The HDCR can be semi-autonomously operated and remotely monitored, which allows it to be comfortably operated by a single technician. To reduce clean room demand and mitigate contamination risk, we have engineered the HDCR to be operable in an entirely closed manner with MicroCNX aseptic connectors allowing for addition or removal of samples from the unit. This allows it to integrate straightforwardly into a closed processing train (i.e. HDCR, chemical or mechanical lysis, chromatography, TFF). The device is operable inside of a standard cell incubator simplifying facility demands. Manufacturing of commercial-grade HDCR units is currently underway using USP Class VI materials for cell contacting components. With these advancements, the HDCR system is nearing readiness for deployment in the CGT community to facilitate process intensification and overcome production bottlenecks.

1177 Non-Viral Persistent Genetic Modification of NK-92 Cells with Non-Integrating S/MAR Vectors for Cancer Immunotherapy

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Natural Killer (NK) cells form part of endogenous tumour immunosurveillance and have powerful cytotoxic activity against cancer cells. The cell line NK-92 is commonly used in preclinical and clinical studies exploring NK-cell based cancer immunotherapies due to its ease of handling in contrast to primary NK cells, making it suitable for off-the-shelf NK cell therapies. Multiple clinical studies have shown the safe administration of irradiated NK-92 cells to cancer patients, but the therapeutical efficacy has been limited so far. To enhance the anti-tumour activity of NK-92 cells, they can be genetically modified to express a chimeric antigen receptor (CAR) binding to a tumour-specific epitope or to express immunomodulating cytokines. Nevertheless, the genetic modification of NK cells has proven to be very challenging. To modify NK-92 cells, researchers rely mainly on transduction with retro- and lentiviruses. As a natural function of NK cells is to defend against viral infections, NK cells react strongly to viral vectors leading to low transduction efficiencies. Furthermore, transduction bears the risk of insertional mutagenesis and limits the size of the transgenes that can be delivered. We have developed a novel DNA Vector platform which contains no viral components and a minimal bacterial backbone; it does not integrate into the target cell's genome but can replicate autonomously and extrachromosomally in the nucleus of dividing cells using human sequences known as Scaffold/Matrix Attachment Regions (S/MAR). Therefore, these

vectors are excellent candidates for the persistent, stable modification of NK cells without disrupting the genomic integrity of the modified cells. We have developed electroporation protocols and modified our S/MAR vectors to determine optimal vector features to improve the genetic modification of NK-92 cells as these cells are notoriously difficult to transfect. This allowed us to generate an NK-92 cell line which stably expresses a reporter gene without altering its molecular phenotype or limiting the killing activity towards K-562 tumour cells compared to the non-modified parental cell line. The expression of the reporter gene was consistent for at least three months and was not influenced by cryogenic cell storage. The treatment of NK-92 cells with small molecules inhibiting the TBK1/IKKε Kinase complex, a central node of cytosolic DNA sensing, before and after transfection improved transfection efficiencies up to threefold for reporter gene vectors. It furthermore enabled the modification of NK-92 cells with CAR-expressing S/MAR vectors and we will describe protocols for the persistent expression of CARs in NK-92 cells using this vector system. These findings are a first step towards the safer stable modification of NK-92 cells for clinical use and provide a foundation to extend the application of our episomally maintained vectors to primary NK cells.

1178 Harvest Clarification of AAV via Depth Filtration and AEX Functionalized Filters

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Adeno-associated virus (AAV) vector-mediated gene delivery has broad therapeutic implications for a wide array of diseases, particularly for ultra-rare genetic disorders. With the recent clinical and commercial success of AAV vectors, new and better technologies are required that enable process strategy simplification, increased efficiency and reduced cost of goods. The first step in the AAV purification process is primary clarification where the target viral vector product is separated from insoluble cell debris and any precipitated host-cell proteins and DNA. In this work, we evaluated a two-stage depth filter train with varying pore sizes for AAV harvest clarification and compared it to a new chromatographic clarifier comprised of a fibrous anion exchange (AEX) chromatography media and a 0.2 μm polyether sulfone (PES) membrane. The AEX-functionalized chromatographic clarifier has the potential for process simplification by compressing primary clarification to a single stage. We further evaluated the clarification efficiency of the two filter trains in both the presence and absence of a nuclease, with the latter offering a cost reduction opportunity in AAV production. The clarification strategies discussed herein provide simple, efficient, and economical solutions to purify AAVs.

1179 Safe and Effective Delivery of Nucleic Acids Using Proteolipid Vehicles Formulated with Fusion-Associated Small Transmembrane Proteins

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Genetic medicines hold great promise to treat a wide array of diseases, yet success in the clinic has been hindered by limitations in the tolerability, scalability, and immunogenicity of current delivery platforms. We sought to overcome these limitations by combining aspects from viral and non-viral platforms to develop a proteolipid vehicle (PLV) that incorporates fusion-associated small transmembrane (FAST) proteins from fusogenic orthoreoviruses into a well-tolerated lipid formulation using a scalable microfluidic mixing approach. We screened a library of FAST recombinants to identify a chimeric FAST protein with enhanced membrane fusion activity. A series of lipid formulations incorporating the chimeric FAST protein were optimized for high nucleic acid encapsulation, charge neutralization, and improved tolerability *in vitro* and *in vivo*. FAST-PLVs administered systemically in mouse and non-human primate models demonstrated broad biodistribution and significantly improved intracellular delivery and expression of messenger RNA (mRNA) and plasmid DNA (pDNA). At high local or systemic doses FAST-PLVs showed low immunogenicity and maintained activity upon repeat dosing over extended periods. We utilized FAST-PLVs to deliver a pDNA follistatin gene therapy *in vivo* that increased circulating levels of follistatin, resulting in significantly increased muscle mass and grip strength. The activity and safety profile of FAST-PLVs make them a promising platform for redosable gene therapies and genetic medicines.

1180 Evidence of AAV Genome Plasmid Concatemerization within E. coli

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For many years, it has been known that in vivo transduction of recombinant adeno-associated virus (AAV) forms circular genome concatemers in animal models through recombination. But the ability of AAV to form these large concatemers within bacteria has been overlooked. The formation of plasmid concatemers in bacterial cells is a natural phenomenon especially in high-copy-number (relaxed type) plasmids. Bacteriophages also commonly undergo rolling circle replication from a ssDNA template, much like the AAV genome, resulting in large, linear DNA segments that are separated by nucleases and packaged separately. In the present study, we identified the presence of rAAV transgene plasmid concatemers formed within in bacteria during plasmid replication. Originally, we noticed a discrepancy between the predicted sequence length and the results from Oxford Nanopore sequencing of the AAV2 ITRs cloned into the UF11 plasmid

backbone. Sequences ranged from one to five times larger than the anticipated plasmid size of 4570 bp, indicating that concatemerization might be present inside the bacterial cells. The UF11 family of plasmids are unique in having CG clamp adjacent to the ITR (18 C repeats preceding 5' ITR and 18 G repeats following the 3' ITR). To understand the significance of the GC clamp in concatemerization, AAV transgene plasmids lacking the CG clamp sequence also were sequenced. In the non-CG clamp AAV plasmids the concatemerization was limited to duplication of the plasmid during bacteria replication, which confirmed this theory. These results suggest that the UF11 CG clamp enhanced plasmid concatemerization in bacteria with over twice the number of copies as compared to plasmids without the GC clamp. Using next generation sequencing, like nanopore, we were able to find evidence of concatemers plasmid that Sanger sequencing methods were not able to detect. AAV plasmid concatemerization affects how we calculate AAV production by transfection: the assumption of a single copy of genome per plasmid is no longer valid.

1181 Astrocyte-Targeted Gene Therapy Demonstrates Efficacy in Two Murine Models of Vanishing White Matter Disease

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Vanishing White Matter Disease (VWM), is a leukodystrophy, a neurodegenerative white matter disorder that most commonly occurs in children. Clinically, it presents with ataxia, spasticity, neurological decline, and seizures which lead to premature death. Currently there are no treatments for VWM. VWM is caused by autosomal recessive, loss of function mutations in the subunits of eukaryotic initiation factor 2B (EIF2B), with pathologic variants in EIF2B5 being the most common. Due to VWM's monogenic nature, it is a good candidate for adeno-associated virus (AAV)-mediated gene replacement therapy. VWM pathology suggests that astrocytes are a critical target for therapy, as their differentiation, morphology, and function is impaired, thus mediating disease progression. Therefore, we designed gene replacement constructs to compare astrocyte-specific or ubiquitous expression. Intracerebroventricular (ICV) injections of our vectors expressing the reporter protein GFP into wild-type mice revealed that our astrocyte-specific reporter constructs (AAV9.GFAP.eGFP and AAV9.gfaABC(1)D.eGFP) achieved appropriate transgene expression in astrocytes; with our AAV9.GFAP.eGFP vector having significantly greater biodistribution throughout the neuroaxis when compared to its truncated, or ubiquitous counterparts (AAV9.gfaABC(1)D.eGFP and AAV9.CAG.eGFP). To evaluate potential therapeutic efficacy, we initially generated three constructs driving expression of the *EIF2B5* transgene. However, due to the size capacity of AAV (4.8kb), the GFAP promoter (2.2kb) in combination with the *EIF2B5* transgene (2.2kb) and necessary regulatory elements, led to an oversized construct, subsequent poor packaging, and low viral titers. Because of significant differences in expression between the full-length and truncated astrocyte promoters in our GFP reporter study, we endeavored to increase biodistribution by generating a tailored promoter that restores crucial sequences from the endogenous GFAP promoter into its truncated gfaABC(1)D counterpart. Thus, we created a **novel**, intermediate gfaABCD1405 promoter, and preliminary studies demonstrate expression comparable to full-length GFAP. We evaluated our four AAV constructs in two murine VWM models, *Eif2b5*^{Arg191His} and *Eif2b5*^{Ile98Met}, which display significant gait deficits, myelin loss, and shortened life span, and are monitoring disease progression using traditional and clinically relevant readouts including magnetic resonance imaging (MRI), electroencephalogram (EEG), and gait analysis. Our current data suggests that our astrocyte-targeted gene therapy is able to delay disease progression, partially rescue body weight, and significantly increase latency to fall on rotarod in both disease models; this is to a significantly greater extent than ubiquitous gene therapy. Overall, we anticipate emergence of a lead astrocyte-targeted gene therapy candidate in which the data will be strengthened through the evaluation of clinically relevant measures in two murine models of disease, allowing for timely translation to the clinic. Our findings support the importance of targeting the underlying disease mechanisms through the modification of the AAV vector design.

1182 Targeting Immunosuppressive Tumor-Associated Macrophages Using Innate T Cells for Enhanced Antitumor Reactivity

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The field of T cell-based and chimeric antigen receptor (CAR)-engineered T (CAR-T) cell- based antitumor immunotherapy has seen substantial developments in the past decade; however, considerable issues, such as graft-versus-host disease (GvHD) and tumor-associated immunosuppression, have proven to be substantial roadblocks to widespread adoption and implementation. Recent developments in innate immune cell-based CAR therapy have opened several doors for the expansion of this therapy, especially as it relates to allogeneic cell sources and solid tumor infiltration. This study establishes in vitro killing assays to examine the TAM-targeting efficacy of MAIT, iNKT, and γδT cells. This study also assesses the antitumor ability of CAR-engineered innate T cells, evaluating their potential adoption for clinical therapies. The in vitro trials presented in this study demonstrate the considerable TAM-killing abilities of all three innate T cell types, and confirm the enhanced antitumor abilities of CAR-engineered innate T cells. The tumor- and TAM-targeting capacity of these innate T cells suggest their potential for antitumor therapy that supplements cytotoxicity with remediation of tumor microenvironment (TME)-immunosuppression.

1183 Development of Adenovirus Serotype 4 Oncolytic and Gene Therapy Vectors as an Alternative to Adenovirus Serotype 5

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Adenoviral vectors are explored for gene therapy and cancer treatment and vaccination. Despite limited applicability caused by preexisting immunity and unfavorable liver tropism, most adenoviral vectors are derived from human Adenovirus 5 (AdV5). We therefore screened an Adenovirus library representing the natural Adenovirus diversity to identify potential alternative adenoviral vector candidates. To compare different AdVs infectivity and AdV mediated and reporter-gene expression we assessed GFP or luciferase activity in variety of cell lines and further tested oncolytic activity using viability assays. Candidate oncolytic vectors were tested on cervical cancer cell mini-organoids. Among many AdV serotypes we found that AdV4 transduced muscle and heart cells more efficiently than AdV5. Moreover, despite low initial transduction efficiency, AdV4 showed oncolytic activity, efficient long lasting gene expression, genome replication and progeny virus production in papillomavirus (HPV) induced tumor organoids. We constructed a first generation AdV4 vector platform by deleting E1 and E3 genes. The E1 and E1-E3-deleted vectors were replication-competent in HEK293 cells, whereas other cancer cell lines including Hela and Siha were not permissive, suggesting that AdV5-E1 can complement for AdV4-E1, enabling the rescue of E1-deleted AdV4. Purified E1, E3 deleted vectors showed similar transduction efficiencies as the parental vector. To increase AdV4 uptake into Papillomavirus induced cancer cells we replaced the AdV4 fiber knob with the AdV5 fiber knob, but pseudotyping did not improve vector infectivity nor replication. Our new AdV4 vector will be further explored for oncolytic or muscle gene therapy and cardiac gene therapy leading the way for the development of further alternative AdV vectors for a broad range of clinical applications.

1184 Reversal of Cardiac Phenotype in a Mouse Model of Friedreich's Ataxia Following Administration of AAV Gene Therapy

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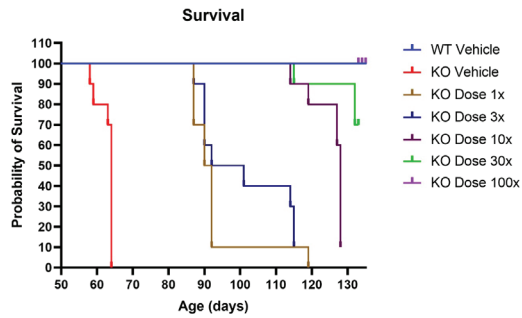
Friedreich's ataxia (FA) is a multisystem autosomal recessive disease characterized by severe and progressive neuropathy accompanied by hypertrophic cardiomyopathy that is frequently fatal. FA is caused by biallelic GAA trinucleotide repeat expansions in the *FXN* gene that reduces expression of the mitochondrial protein frataxin. We investigated the potential to reverse or prevent progression of the cardiac phenotype in a mouse model of FA, by transferring the human version of the codon-optimized *FXN* gene using an adeno-associated viral vector serotype 8 (AAV8). This AAV construct has a phosphoglycerate kinase (PGK) promoter (AAV-PGK-hFXN) to control the frataxin expression. The cardiac and skeletal muscle-specific frataxin knockout mouse model (KO) has progressive cardiac deficits leading to early mortality. In this study, KO mice received a single intravenous injection of either vehicle or AAV-PGK-hFXN at escalating dose levels (1x, 3x, 10x, 30x, and 100x) to evaluate efficacy and safety at 4- or 12-weeks post-dose. Administration of AAV-PGK-hFXN to KO mice resulted in a dose-dependent reduction in early mortality compared with vehicle-treated KO mice (Fig. 1). In addition, AAV-treated KO mice had statistically significant improvements in ejection fraction and left ventricular mass normalized to body weight ratios compared to mice that received vehicle. Serum biomarkers for cardiac injury including myosin light chain (Myl) and cardiac troponin (cTnI), were 24- and 21-fold higher in vehicle-treated KO mice compared to C57BL/6J wild type mice and were significantly reduced in KO mice following AAV-PGK-hFXN treatment. Transgene DNA was detected in the hearts of AAV-treated mice in a dose-dependent manner. Following the highest dose (100x), exogenous frataxin expression was 14-fold higher in the hearts and ~100-fold higher in the livers of KO mice compared with endogenous levels in WT mice. However, no significant differences were noted in terminal serum alanine aminotransferase or aspartate aminotransferase levels at 4 weeks post-dose when comparing all AAV-treated KO mice to vehicle-treated WT mice. Reduction of myocardial lesions following AAV treatment was consistent with the improvement in cardiac function and the reduction of cardiac injury biomarkers. At 12 weeks post-dose, KO mice that received the two highest doses of AAV-PGK-hFXN, displayed slight to minimal axonal degeneration in the sciatic nerve (30x, 3/10; 100x, 10/10) and, in a few mice, signs of neurodegeneration in the dorsal root ganglion (100x, 1/10). None of these histological changes were accompanied by any clinical manifestations. In addition, the livers of KO mice treated with AAV-PGK-hFXN displayed Kupffer cell hypertrophy and minimal hepatocyte necrosis. These liver changes are commonly associated with AAV gene therapies, are likely related to gene expression, and are considered non-adverse given the low incidence of observations. Overall, dose-dependent efficacy was observed in AAV-PGK-hFXN-treated KO mice compared with vehicle-treated mice based on survival, cardiac function, cardiac injury biomarkers, and histology. These data support the use AAV-based gene transfer as a promising approach to treat patients with FA cardiomyopathy.

Figure 1:



1185 Extracellular Vesicles of Heart-Derived Cells but Not Bone Marrow or Umbilical Cord-Derived Mesenchymal Stem Cells Attenuate NLRP3 Inflammasome Activation in Macrophages

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Background: Intramyocardial injection of heart-derived cells (HDCs) or mesenchymal stem cells (bone marrow, BM-MSCs or umbilical cord, UC-MSCs) has been demonstrated to improve cardiac function in animal models of ischemic heart disease (IHD). This effect in part was attributed to anti-inflammatory microRNA (miRNA) cargo within the extracellular vesicles (EVs) produced by the injected cells. However, it is not known if EVs modulate NLRP3 inflammasome, an innate immune protein complex implicated in the pathology of heart disease. **Objective:** We sought to assess and compare the effect of 3 stem cell derived EVs on NLRP3 inflammasome activation in macrophages; a critical pro-inflammatory cell type. **Methods:** EVs were isolated from conditioned media (ultracentrifugation) and EV miRNA cargo was profiled using multiplex fluorescent miRNA detection (Nanostring). The functional enrichment analysis and miRNA target prediction were performed using bioinformatic tools (Tam 2.0 & miRWalk v3). Monocytes (THP-1) were differentiated into macrophages with phorbol-myristate acetate (PMA; 3 days) and treated with EVs (20 hours) at 3 dose levels (Low:1E+10, Mid:2E+10, High:4E+10 EVs/mL) before inducing NLRP3 inflammasome (lipopolysaccharide; 4 hours & nigericin; 1 hour). The activation of NLRP3 inflammasome was assessed by measuring caspase-1 activity (Caspase-Glo®1 assay), IL-18 & IL-1β levels (ELISA) in the culture supernatants. **Results:** None of the doses of BM- or UC-MSC EVs showed an inhibitory effect on caspase-1 activity. While low or mid doses of HDC EVs failed to show an effect, a high dose (4E+10 EVs/mL) significantly attenuated caspase-1 activity vs. control group (67% lower, n=4-6, $p<0.0001$). Further, EV treated macrophages showed a significant reduction in IL-18 (623±8 vs.1462±43 pg/mL, $p=0.0025$) and a trend towards reduction in IL-1β levels (1292±115 vs. 2535±115 pg/mL, $p=0.08$). However, neither BM- or UC-MSC EVs showed an effect on IL-18 or IL-1β levels. Interestingly, the bioinformatic analysis revealed HDC EVs were enriched with 22 distinct anti-inflammatory miRNAs, of which 4 miRNAs (miR-181a, miR-100, miR-21, miR-22) were predicted to inhibit NLRP3 inflammasome. **Conclusions:** HDC but not BM- or UC-MSC EVs suppress NLRP3 inflammasome in macrophages and this effect in part is possibly mediated via transfer of highly enriched anti-inflammatory microRNA cargo. These preliminary findings prompted us to focus on validating candidate anti-NLRP3 miRNAs in HDC EVs and assessing their immunomodulatory effects using preclinical models.

1186 Strategic Formulation Development for AAV Delivered Gene Therapies-A Case Study

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Formulation optimization (e.g., choice of buffer, pH, excipients) is crucial to developing and stabilizing viral vectors as delivery vehicles for genomic drug products. Phosphate buffers have been widely used for formulating viral vectors for gene therapy due to their well-known clinical compatibility and desired pH range for biopharmaceuticals. However, phosphate buffer is known to show a considerable pH drop in frozen state, which might scale with volume, irrespective of the buffer concentration. For frozen AAV-based drug product (DP), the freeze/thaw cycling during manufacturing and/or storage and handling with associated pH drop may negatively impact the product critical quality attributes (CQAs). To protect AAV-based DPs from freeze/thaw stress, sucrose can be used as a cryoprotectant. In this study, we explored varying sucrose concentrations and their effectiveness in preventing pH drop mediated degradation of AAVs in a phosphate and a non-phosphate buffer formulation, respectively. This work demonstrates strategic selection of buffer (phosphate/non-phosphate) and other excipients (e.g., sucrose) to enable optimal stability of the DP. Further, we present an analytical method (free DNA assay) that can be utilized in optimizing excipient concentration to manage osmolality, density, and endotoxin contributions, for ocular and central nervous system (CNS) routes of administration.

1187 Identification of the Translational Strategy of MAAP and Its Function in AAV Life Cycle

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Adeno-associated viruses (AAVs) are small ssDNA viruses packaging a single stranded genome of 4.7 kb. The left genome encodes 2 large and 2 small replication (Rep)-important nonstructural proteins, Rep78/68 and Rep52/40. The right genome encodes 3 capsid (Cap) proteins (VP1, VP2, and VP3) and two 2 auxiliary small nonstructural proteins, assembly-activating protein (AAP) and membrane-associated accessary protein (MAAP). MAAP is translated from a frameshifted open-reading frame (ORF) located in the VP1 unique coding region. During recombinant AAV (rAAV) production, MAAP played a role in the production of rAAV and/or the regulation of rAAV release from the cell. An additional role of MAAP in genome packaging has also been attributed. However, the exact role of the MAAP in AAV life cycle and its expression strategy remain elusive. Here, we investigated the expression profile and the function of MAAP in the life cycle of both wild-type (wt)AAV2 and wtAAV5, which are phylogenetically distinct serotypes. We found that both AAV2 and AAV5 MAAPs are expressed from *Cap* transcribing mRNA that encodes VP2 and VP3, which is spliced from the major splicing acceptor site (A2). While we confirmed the CTG site for translation of AAV2 MAAP, the ATT codon immediately after the A2 splice site was used to initiate translation of AAV5 MAAP. When MAAP was knocked out in an AAV2 infectious clone, the produced MAAP KO AAV2 mutant tended to be retained

in the cell, whereas virus replication is not affected significantly. Surprisingly, the decrease in virus release was not reinstalled by providing MAAP in *trans* either with plasmid transfection or inducible expression. Interestingly, when MAAP was complemented in *trans*, viral DNA replication was significantly decreased, which is consistent with a decrease in virus replication and expression of viral mRNAs and proteins, suggesting that MAAP may play an additional role in virus life cycle. Importantly, during late phase of wtAAV2 infection, MAAP localized in the same compartment with the capsid in the cytoplasm, which is close to cell outer membrane, providing evidence of possible association of MAAP with capsid during virus release. However, MAAP did not exhibit any colocalization with TGN or ESCRT pathway during wtAAV2 infection, suggesting that a different egress pathway is used for MAAP to escort capsid during virus release. In summary, MAAP is a novel AAV protein translated from the same mRNA encoding VP2 and VP3. Ectopic expression of MAAP decreases viral DNA replication, mRNA transcription, and protein expression. The ablation of MAAP during wtAAV infection causes a significant delay in capsid release; however, the choice of the virus egress pathway is not affected by MAAP.

1188 Development of a Lipid Library for the Optimized Delivery of Self Amplifying RNA

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The self-amplifying RNA (saRNA) platform is an attractive genetic strategy due to its ability to express multiple gene of interests (GOI), higher efficiency in non-dividing cells compared to conventional mRNA, transient activity, and biodegradability. Furthermore, its inherent replication ability distinguishes it from conventional mRNA and could alter the therapeutic window by facilitating higher and longer protein production at lower doses. The major challenge of any nucleic acid (NA) and in particular RNA-based platform is to deliver the molecules to the target cell and subsequently to the cytosol. RNA is sensitive to ribonuclease degradation and NAs in general are unable to cross the cell membrane barrier due to their negative charge. In addition, saRNA specifically is relatively large (over 8kb). Various delivery systems have been developed to protect NAs and shield their negative charge to allow intracellular delivery at the site of action. Lipid nanoparticles (LNPs) have emerged as a promising delivery approach. These are spherical particles formulated when lipids interact with RNA molecules, forming a stable complex protecting NAs against nucleases. Current standard LNPs consists of four lipids: an ionizable cationic lipid, a phospholipid, a sterol, and a polyethylene glycol (PEG) lipid. However, the LNP safety profile and the use of these vehicles for the efficient delivery of large molecules such as saRNA still requires optimization. This study aims to develop LNP-based delivery vehicles which efficiently complex saRNA, thereby ensuring protection against extracellular degradation yet enabling efficient cell uptake and subsequent endosomal escape. Therefore, twenty novel ionizable cationic lipids were designed and synthesized intending the formulation of twenty next-generation LNPs. Initial optimization experiments using a reference ionizable lipid together with the auxiliary lipids were performed to establish standard formulation conditions to test the novel ionizable lipids. A rigorous characterization of the LNPs formulated for each novel ionizable lipid has been performed to determine the size distribution, polydispersity index (PI), charge, and encapsulation efficiency (Enc%). Several exclusion criteria of the physio-chemical properties were chosen to eliminate formulation prior to the in vivo trails: size ≥ 150 nm, PI ≥ 0.3, charge [-20 mv to +20 mv], and Enc% ≥ 80%. All novel formulated LNPs successfully achieved this target product profile. Subsequently, LNPs were intramuscularly administered in mice. To assess saRNA expression efficiency, luciferase-induced bioluminescence was measured via non-invasive in vivo imaging (IVIS) over the course of 20 days. Remarkably, all novel LNPs enabled a positive expression pattern ranging between a 4-6-fold change in log10 scale compared to negative controls (untreated, naked saRNA, and LNPs without ionizable lipid). Next, six formulations with a significantly different total expression (AUC) were identified, either for their higher expression or longevity in comparison to the reference LNPs (positive controls). Accordingly, we report that structural differences of the novel ionizable lipids, including the number of side chains, length of the linkers, as well as the inclusion of functional groups, have a substantial influence on the physio-chemical and biological characteristics of the LNPs.

1189 Development of an *In Vitro* Potency Assay for Neuropeptide-Expressing AAV Vectors Targeting Neurological Diseases

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Neuropeptide receptors are gaining more and more attention as therapeutic targets due to their modulatory effects on neuronal excitability. However, small molecule agonists for neuropeptide receptors frequently induce unwanted effects due to activation of receptors outside of the target area. Focal application of viral vector-based gene therapies expressing neuropeptides overcomes these obstacles and therefore offer interesting alternatives for long-term treatment. Determining vector potency as a critical quality attribute is important when developing gene therapies and requires a reliable assay for lot release and market approval. With the recent mitigations of FDA regulations concerning the requirements for animal testing, the need to develop robust *in vitro* assays is becoming even more urgent. This may be challenging due to the lack of well characterized human cell lines for specific cell or tissue types. Moreover, available cell lines may not be transduced well by specific AAV serotypes, or lack factors needed for the proper expression or processing of vector-transduced proteins. Using a human neuroblastoma cell line, we have developed an all-in-one biopotency assay that allows parallel determination of RNA, and fully processed neuropeptide expression, as well as viral entry in the same infected microplate well. We compared several preprodynorphin-expressing AAV vectors between our newly developed *in vitro* potency assay and the established *in vivo* assay. Expression levels of fully processed, mature dynorphin B in the cell-based assay correlated with expression levels in vector-transduced mouse brain tissue. The novel human neuronal cell-based *in vitro* assay will facilitate vector screening, allow AAV batch to batch validation, and reduce the need for animals in potency testing.

1190 Nonclinical Development Pathway of a DNA-Encoded Bispecific T Cell Antibody Targeting HER2

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Bispecific antibodies have revolutionized the treatment of human cancers with significant advances in clinical development surging in the past decade. However, conventional recombinant therapeutics have practical limitations including *in vivo* durability, complex and costly manufacturing, and poor stability. Here, we describe the nonclinical program of a DNA-based medicine (INO-B002) encoding a bispecific antibody targeting epidermal growth factor receptor 2 (ERBB2; HER2) tumor antigen on cancer cells and CD3 on T cells (dBTA-HER2CD3). DNA medicines are highly stable, easier to manufacture, and are expressed *in vivo* for months following a single pDNA treatment delivered with electroporation (EP) technology in preclinical models. Following delivery to muscle tissue of INO-B002, the *in vivo* produced- dBTA-HER2CD3 binds to human CD3 but does not bind to cynomolgus monkey or mouse CD3, thus no pharmacologically relevant animal species exists for testing the toxicity of dBTA-HER2CD3. Therefore, following a pre-IND meeting with the FDA, we implemented a strategy that utilized an *in vitro* approach to assess nonclinical safety. We demonstrate that dBTA-HER2CD3 activates T cells, induces cytokine production, and specifically kills HER2-expressing cancer cells *in vitro.* We observed differential activity in co-cultures with HER2-expressing cancer cells and PBMCs compared to co-cultures with normal cells that express low levels of HER2. *In vivo,* dBTA-HER2CD3 is expressed with measurable levels of functional dBTA detected in both mice and pigs. In addition, dBTA-HER2CD3 controlled tumor growth and improved animal survival in a dose dependent manner in a humanized mouse model. The data collected from these studies has guided the identification of the minimum anticipated biological effect level (MABEL) of dBTA-HER2CD3, which may be used in combination with preclinical pharmacokinetic (PK) studies to determine the starting pDNA dose for a first-in-human (FIH) Phase 1 trial.

1191 Immunosuppression to Inhibit Capsid-Specific Humoral Immune Responses in High-Dose AAV Gene Therapy in Cynomolgus Macaques

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Innate and adaptive immune responses pose a potential limitation for safe and effective gene therapy using recombinant adeno associated viruses (rAAVs). High-dose systemic delivery of rAAV has, in some cases, resulted in serious adverse events such as thrombotic microangiopathy (TMA) and atypical hemolytic uremic syndrome (aHUS) leading to kidney damage due to complement activation. A potential cause is the binding of IgG and IgM anti-capsid antibodies to rAAV vectors in circulation, to form rAAV-antibody complexes, triggering activation of the complement pathway. Thus, immunosuppression (IS) to inhibit the anti-capsid IgM and IgG responses may be useful to mitigate complement-mediated adverse events in high dose gene therapy. Here, immunosuppression with prednisolone, rapamycin and rituximab-pvvr was evaluated for the ability to decrease the development of anti-capsid IgM and IgG. An investigational AAVhu37 gene therapy vector expressing human microdystrophin (μDys) for the treatment of Duchenne muscular dystrophy was dosed at 2e14 VG/kg in male cynomolgus macaques. Pre-screened animals seronegative for both anti-AAVhu37 neutralizing antibodies (NAb) and for AAVhu37 anti-capsid IgGs were included. Animals were divided into 5 study groups. Animals in Group 1 did not receive either IS or rAAV. Animals in Group 2 received rAAV alone. Animals in Groups 3, 4 and 5 received rAAV and IS of prednisolone, prednisolone/rapamycin and prednisolone/rapamycin/rituximab-pvvr, respectively. Plasma, serum, and whole blood were assessed at different time points to evaluate immune endpoints such as anti-capsid IgM, anti-capsid IgG and NAb levels, platelet numbers and complement protein levels. Whole blood was monitored for B cell numbers and rapamycin levels throughout the study. Peripheral B cells were depleted by rituximab-pvvr, and rapamycin levels were above the target level of 12ng/ml for the duration of the study. A transient elevation in liver enzymes (ALT, AST) was observed in all dosed groups four days after vector dosing. The combination of prednisolone, rapamycin and rituximab-pvvr (Group 5) demonstrated a significant decrease in the anti-capsid IgM, IgG and NAb levels compared to the no IS group (Group 2). Four days after vector administration, Group 5 also exhibited significantly higher vector genome DNA levels in the serum, possibly due to the lower anti-capsid antibody levels. A transient increase in complement (C5b9 and C3a) proteins and a transient decrease in platelet numbers were observed in only a subset of animals from all dosed groups within the first week. The combination of prednisolone, rapamycin and rituximab-pvvr was well tolerated and reduced anti-capsid IgM and IgG following high-dose rAAV gene therapy.

1192 The Evolution of a Manufacturing Process for an Integrating AAV Vector: Understanding the Impact of Upstream and Downstream Process Changes

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hLB-001 is an AAV drug product which is being utilized in the clinic for the treatment of pediatric patients with methylmalonic acidemia characterized by methylmalonyl-CoA mutase gene (MMUT) mutations. hLB-001 uses GeneRide™ technology and incorporates homology arms in the vector design to precisely integrate a corrective MMUT gene into the albumin locus of targeted cells through homologous

recombination. Selective advantage is then expected to promote the proliferation of edited cells that express the functional protein. The first generation manufacturing process for hLB-001, which we refer to as Process A, was used to generate an initial batch of product to be used as reference material. Process B is the result of several major upstream changes to boost titer and reduce CoGs, namely through the use of a two-plasmid transfection system and a different transfection reagent. Process C fully transitions Process B from CsCl ultracentrifugation to chromatography-based enrichment in anticipation of large-scale manufacturing. Several assays were used to elucidate the impact of these process changes. Digital Droplet PCR (ddPCR) with primer/probes targeting the MMUT transgene was used to measure total AAV productivity in the bioreactor. Analytical Ultracentrifugation (AUC) was used to determine the percentage of both full and partially filled capsids. The heterogeneity of packaged vector genomes was assessed with two different readouts from Nanopore sequencing: the percentage of reads mapped to different reference sequences, and a measurement of the percentage of full-length vs. fragmented transgenes based on coverage across the transgene. Finally, two different biological potency assays were used to quantify the fused mRNA expression and enzymatic activity of the therapeutic protein. Upstream process changes were found to result in the most dramatic difference in the product profile, while the transition from CsCl enrichment to an anion exchange chromatography enrichment step had a relatively minor impact. Nanopore sequencing assisted in the identification of critical factors in the upstream process that increased the full-length packaging of transgenes into AAV vectors. The results of this study highlight the importance of well developed assays that can detect changes in critical quality attributes and will build a robust understanding of how process changes affect the drug's product profile.

1194 Lentiviral Vectors with Regulated Expression to Treat X-Linked Lymphoproliferative Disease

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Introduction: XLP1 is a primary immunodeficiency caused by loss of function mutations in the *SH2D1A* gene, which dysregulates an adaptor molecule, SAP, involved in SLAM-family immune cell signaling. Patients exhibit severe immune impairment - exacerbated following Epstein-Barr Virus infection - which leads to hemophagocytic lymphohistiocytosis, hypogammaglobulinemia, and lymphoma. XLP1 is a monogenic disorder and therefore an excellent candidate for an autologous hematopoietic stem cell (HSC) gene therapy. A previous XLP1 lentiviral vector (LV) gene therapy approach utilized a ubiquitously expressing EFS promoter to encode for the SAP protein. The ubiquitous expression pattern of the EFS LV failed to emulate endogenous regulation of the integrated transgene and suffered from impaired skewing of HSC differentiation. To overcome detriments of ectopic expression, we experimentally elucidated genomic elements that regulate the *SH2D1A* promoter to rationally design LVs that emulate the lineage and temporal specific control of SAP within T, NK, and NKT cells. **Methods:** Elements responsible for regulated expression of the endogenous *SH2D1A* locus, were elucidated by leveraging four genome-wide enhancer databases (ENCODE, VISTA, FANTOM, and Ensembl). We refined each putative element using the UCSC genome browser to minimize sequence length and retain transcription factor binding, epigenetic modifications, enhancer RNA activity, and chromatin interactions. Each element was cloned upstream of the minimal endogenous *SH2D1A* promoter to drive expression of a GFP reporter gene and a unique barcode for multiplexed screening. On-target populations (primary T, NK, and NKT cells) and off-target populations (primary monocytes and B-lymphoblastoid cell lines) were transduced with the barcoded LVs and assessed for their relative enhancer activity by next generation sequencing. Enhancer elements were combined to augment synergistic lineage-specific expression. Expression was assessed *via* GFP mean fluorescent intensity (MFI) in on- and off-target populations, compared to the minimal *SH2D1A* promoter or the ubiquitous EFS promoter. Finally, enhancer temporal expression was confirmed by transducing mobilized peripheral blood CD34+ HSCs with the lead candidate LV to assess GFP+ MFI across T-cell development within the Artificial Thymic Organoid (ATO) system. **Results:** We identified 34 regulatory elements within 200 kb of the human *SH2D1A* gene. Six elements - tested individually - enhanced the minimal promoter activity in T, NK, and NKT cells up to 2-fold. The top 3 enhancer candidates were combined to synergistically **increase expression in on-target populations up to 4-fold** compared to the basal *SH2D1A* promoter and up to 2-fold compared to the previous pre-clinical EFS LV. Furthermore, the XLP-LV demonstrates **no off-target activity**, in contrast to the EFS LV, which contained 3.5-fold greater off-target expression over the basal *SH2D1A* promoter. We also demonstrated that **XLP-LVs mimic temporal expression patterns of SAP** protein throughout T-cell development in ATOs. **Conclusion:** This project delineates not only the first lineage specific LV treatment for XLP1 but also a universal method for the quick identification and incorporation of enhancers into LVs that achieve temporal and lineage specific expression of a target gene. We identified several enhancers of *SH2D1A* that, in combination, increase promoter activity by 4-fold in on-target populations (2-fold greater than the previous EFS LV). The lead XLP-LV also maintains zero off-target expression, unlike the pre-clinical EFS LV counterpart. Current efforts are underway to assess functional restoration of SAP deficient cells in an *in vivo* model.

1195 Programmable Protein Stabilization with Language Model-Derived Peptide Guides

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Post-translational modifications (PTMs) of proteins increase functional diversity, but dysregulated alterations can lead to several diseases such as cancer and autoimmune disorders. Specifically, deubiquitination counteracts proteasomal degradation by removing ubiquitin protein chains, thus having several regulatory implications on DNA repair, cell differentiation, and folding catalysis processes. While targeted protein degradation modalities have been developed, a counterpart "targeted protein stabilization" of key regulatory proteins represents a powerful tool to rescue improperly ubiquitin-tagged proteins. Unfortunately, a

significant set of these proteins are considered undruggable by standard small molecule-based therapeutics, due to the lack of binding site accessibility and their disordered nature. As a more programmable, genetically-encoded strategy, designing functional peptide-guided stabilizers provides a unique opportunity for therapeutic interventions. To design a modular architecture for protein stabilization, we leverage protein language models, trained on millions of natural amino acid sequences, to capture diverse biochemical, structural, and functional features of input protein sequences. Specifically, we fine-tune pre-trained protein language models for the specific task of conditionally generating binding peptides provided a target amino acid sequence. We experimentally fuse model-derived peptides to deubiquitinase (DUBs) domains, generating an architecture we term "deubiquibodies" (duAbs), and systematically identify peptide-guided constructs that exhibit robust intracellular deubiquitination in human cells across diverse targets. We further show that our duAbs have negligible off-target effects via whole-cell proteomics and demonstrate re-stabilization of fusion oncoproteins in pediatric cancer cell models, inducing targeted tumor cell death. Overall, our results motivate *in vivo* characterization of our genetically-encoded therapeutic platform for downstream clinical translation.

1196 Novel Strategies to Enhance the Efficacy of CAR T-cell Immunotherapy in Glioblastoma

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Glioblastoma (GBM) is an extremely aggressive primary brain tumor, with an overall survival of about a year after diagnosis. Conventional therapies have not resulted in major improvements in the survival outcomes of patients with GBM, so increasing efforts are being devoted to develop new therapeutic strategies, particularly in the flourishing field of chimeric antigen receptor (CAR) T-cell immunotherapy. However, CAR T-cell therapy in GBM, and in solid tumors in general, faces several challenges including the high level of heterogeneity that can lead to antigen escape, and the immunosuppressive tumor microenvironment (TME). To overcome the risk of antigen escape we propose the use of dual CAR T-cells that simultaneously recognize a novel but "conventional" tumor associated antigen (TAA) recently identified by our group and expressed on the cell surface, and an "unconventional" target antigen expressed in the tumor extracellular matrix (ECM). TAAs in the ECM are generally more accessible, abundant and stable than those located on the cell surface and provide an advantage in terms of antigen escape. We have demonstrated the specificity and functional activity of the mono-specific CAR T-cells *in vitro*, and their therapeutic efficiency *in vivo*, in murine and human GBM models, and we have encouraging results with different modalities we designed for the dual antigen targeting. In parallel, to overcome the obstacle of the TME, we are working on different combination strategies to enhance the efficacy and persistence of the CAR T-cells. These include the combination with immunomodulatory agents that can revert the immunosuppressive milieu and reinvigorate T-cell effector mechanisms. In order to ensure a localized and safe expression of the agents in the tumor site, one approach consists of using a lipid-nanoparticle (LNP) mRNA based tool for *in vivo* targeted expression with promising preliminary results. In another strategy, we evaluated a potential neoadjuvant therapy based on NanoGhost loaded with an anti-GBM drug and expressing an immunomodulatory receptor. Finally, to enhance CAR T-cell metabolic fitness and overcome the nutrient-deprived TME, we have engineered "metabolically superior" cells capable of using an alternative source of energy. Although GBM is a challenging candidate for CAR T-cell immunotherapy, we consider that the combination of dual CAR T-cells armored with new metabolic abilities, and nanomedicines that can remodel the TME represents a promising therapeutic approach.

1197 Stable Expression of a Secreted HIV Decoy Receptor after *In Vivo* HSC Transduction in Mice and NHPs: Safety and Efficacy in Protection from SHIV

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The HIV global pandemic continues with an annual incidence of 1.5 million new HIV infections per year. New approaches are needed to overcome issues of pre-exposure prophylaxis as well as therapy of established HIV infection. Autologous hematopoietic stem cell (HSC) gene therapy is a promising strategy for the treatment of HIV/AIDS. However, despite encouraging preclinical and clinical results, current *ex vivo* HSC gene therapy protocols are complex, expensive and associated with significant toxicities. We aim to develop an *in vivo* hematopoietic stem cells (HSC) gene therapy approach for persistent control/protection of HIV-1 infection based on the stable expression of a secreted decoy protein for HIV receptors CD4 and CCR5 (eCD4-Ig) from blood cells. HSCs in mice and a rhesus macaque were mobilized from the bone marrow and transduced *in vivo* by an intravenous injection of HSC-tropic, integrating HDAd5/35++ vectors expressing rhesus eCD4-Ig. *In vivo* HSC transduction/selection resulted in stable serum eCD4-Ig levels of ~100 μg/ml (mice, 32 weeks) and >20 μg/ml (rhesus 57 weeks) with IC_{50}s of 1 μg/ml measured by an HIV neutralization assay. The *in vivo* HSC transduction approach and high level eCD4-Ig serum levels were well tolerated without abnormalities in hematological parameters. After high- dose, intravenous SHIV.D challenge of rhesus macaques injected with HDAd-eCD4-Ig or a control HDAd5/35++ vector, peak plasma viral load levels were ~50-fold lower in the eCD4-Ig expressing animal. Furthermore, the viral load was lower in tissues with the highest eCD4-Ig expression, specifically the spleen and lymph nodes. SHIV.D challenge triggered a selective expansion of transduced $CD4^+CCR5^+$ cells, thereby increasing serum eCD4-Ig levels. The latter, however, broke immune tolerance

and triggered anti-eCD4-Ig antibody responses, which could have contributed to the inability to eliminate SHIV.D. Our data will guide us in the improvement of the *in vivo* approach. Clearly, our conclusions need to be validated in larger animal cohorts.

1198 Small Nuclear RNA-Mediated Exon 51 Skipping AAV9 Gene Therapy for the Treatment of Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is an X-linked fatal neuromuscular disorder characterized by progressive muscle weakness and loss of ambulation by teenage years, affecting ~1:5000 males aged 5-9[1]. DMD is caused by mutations which shift the transcript reading frame of the *DMD* gene and result in little to no dystrophin protein. Approved exon skipping antisense oligonucleotides (AON) restore the *DMD* reading frame, creating a slightly internally truncated but functional dystrophin protein; however, these treatments show low levels of exon skipping and dystrophin production in skeletal muscle and heart due to poor tissue uptake in disease-relevant tissues. AAV9 gene therapy carrying multiple small nuclear RNAs (snRNAs) programmed to target one or more splice regulatory sequences simultaneously with high precision and potency has shown great therapeutic potential with unprecedented levels of exon skipping in DMD patients[2,3]. The challenge in viral packaging of snRNA genomes is that the repetitive snRNA sequences are prone to truncations/recombinations leading to low titers and inconsistent transgene expression. To overcome these challenges, we have developed a novel snRNA packaging strategy, which minimizes repetitive sequences within the genome and leads to high titer, full-length self-complementary virus with the intended genome as confirmed by long-read sequencing. Using this packaging strategy, we engineered snRNAs to target two different exon splicing enhancer sequences within exon 51 of *DMD* and delivered this construct to patient-derived cells and del52hDMD/*mdx* mice by intramuscular (IM) or intravenous (IV) injection. We report dose-dependent exon 51 skipping up to 100% in patient-derived cardiomyocytes and myotubes and upregulation of dystrophin expression. Four weeks after IM treatment, del52hDMD/*mdx* mice show up to 86% exon 51 skipping in skeletal muscle, and 3 weeks after IV treatment up to 73% in skeletal muscle and 90% in heart. Importantly, dystrophin protein levels were upregulated in skeletal and heart tissues in del52hDMD/*mdx* mice with improvements in muscle pathology observed with IM injections. Further, IV delivery of our construct led to lowering of serum creatine kinase (CK) and AST/ALT levels implying reduced muscle damage upon treatment. In summary, our novel designs allow for high-titer viral packaging of full-length AAV genomes carrying multiple highly expressing snRNA cassettes which we show to effectively skip DMD exon 51 and which can be rapidly deployed to therapeutically skip other *DMD* exons by switching antisense targeting sequences. 1. Zhang et al., Neuroepidemiology 2021. 2. Simmons et al., Mol. Ther. Methods Clin. Dev. 2021. 3. Megan Waldrop, ASGCT 2022.

1199 Development of Strategy for Multi-Dose Gene Therapy in Murine Model of Glycogen Storage Disease Type Ia

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Background: Glycogen storage disease type Ia (GSD-Ia) is caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC), an enzyme that catalyzes the hydrolysis of glucose-6-phosphate to glucose in the terminal step of glycogenolysis and gluconeogenesis. Patients with GSD-Ia manifest severe fasting hypoglycemia, metabolic disturbances, and slowed growth with delayed puberty onset. Previously, a recombinant adeno-associated virus (rAAV)-mediated gene therapy had demonstrated its ability to correct fasting hypoglycemia as well as hepatic abnormalities in model animals of GSD-Ia. The study's results led to human clinical trials started in 2017. While the phase 1/2 clinical trial made great strides in the clinical treatment for adult GSD-Ia patients, the newborn and young patients still need to manage the severe phenotype daily. Gene therapy for infant patients could be the ultimate treatment for these life-threatening symptoms. However, the efficacy of this vector when administered at a newborn age has yet to be fully investigated. Previously, it has been shown that transduced rAAV vectors are gradually diluted out or partially lost in the livers of young animals due to the high rate of hepatocyte proliferation associated with liver growth. Thus, 2 separate doses of this vector, newborn and adult, may prove to be the most advantageous method for improving gene therapy in liver directed models. In this perspective, we evaluate the use of endogenous growth factors linked with hepatocyte proliferation to determine optimal age of gene therapy vector administration to minimize vector dilution with GSD-Ia knock-out mice. **Methods:** In this study, we used a GSD-Ia mouse model with complete knockout of G6PC. rAAV vector expressing G6PC was administered in neonatal mice. To gage liver growth rate in the mice we analyzed the growth factors that play major roles in hepatocyte proliferation: growth hormone (GH), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), and hepatocyte growth factor (HGF). We administered 2nd dose of AAV gene therapy vector based on the concentration of the growth factors and then examined the efficacy of the vector by assessing fasting glucose levels, G6PC gene expression, and vector copy numbers in the livers of treated GSD-Ia KO mice. **Results:** We show that after rAAV administration in neonatal mice, survival rate of GSD-Ia KO mice is greatly increased but still have slowed growth. GH, EGF, TGF-α, and HGF serum levels were assessed at 2, 4, 6, 9, and 12 weeks and compared with the growth rate of the KO mice to estimate hepatocyte proliferation rate. Additionally, we observed low fasted glucose levels as early as 6 weeks of age in KO mice, indicating lower efficacy of the vector at this time point. Vector copy numbers and gene expression of G6PC were quantified following the fasting glucose test in the various age groups to evaluate the strategy to determine AAV vector administration age. **Conclusion:** In severe congenital liver diseases, such as GSD-Ia, therapeutics including gene

therapy in newborns would greatly improve the quality of life for patients given the extreme difficulty to properly manage symptoms in infants. This may require a second dose at adult age and determining the optimal timing of a second dose of rAAV will be critical for higher long-term efficacy of the treatment. This study gives examines the correlation of proliferation biomarkers and liver growth rates to shed more light on this concern.

1200 Base-Editing as a Safe and Highly Effective Alternative Treatment for X-SCID Compared to CRISPR-Cas9 Nuclease Editing with an AAV Donor

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Genome editing offers great potential for improved safety and efficacy for *ex-vivo* hematopoietic stem/progenitor cell (HSPC) gene editing, including for the treatment of Inborn errors of immunity (IEI) and other blood disorders. When considering the safety of correcting mutations, different gene editing approaches have advantages and caveats. For example, targeted integration (TI) using exogenous DNA donors following CRISPR-Cas9 nuclease mediated double strand DNA breaks (DSB) via homology-mediated repair (HDR) has demonstrated high TI efficiencies for correction of multiple IEIs. However, DSBs and DNA damage responses (DDR) following DSBs may be compounded by exposure to DNA donors, potentially impairing HSPC fitness and long-term engraftment. Here we compare the safety and efficacy of correcting an *IL2RG* mutation in HSPCs from a patient with X-linked Severe Combined Immunodeficiency (X-SCID). To treat X-SCID, current *ex vivo* HSPC gene therapy uses integrating retroviral vectors. While this treatment provides substantial clinical benefit, insertional oncogenesis remains a risk. We therefore sought to revert the *IL2RG* mutation using a DSB/HDR approach (GE) with CRISPR-Cas9 and an AAV donor compared to base-editing using an Adenine Base Editor (ABE8e-SpCas9). To make this comparison, GE and BE XSCID HSPCs were processed with CRISPR-Cas9 nuclease and an AAV6-IL2RG donor with i53 (favors HDR) and Genetic Suppressor Element (GSE, dampen DNA damage response) or ABE-8eSpWT base-edited HSPCs, which were transplanted into immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg mice pups. We assessed engraftment capabilities following editing since this is a major concern for gene targeted HSPCs, especially following DSBs. Transplanted mice were harvested at 16-24 weeks, and engraftment rates (hCD45+ cells) were similar between GE (with optimal addition of GSE) and BE transplanted mice (GE: 13.01% [SD: 7.81], BE: 13.52% [SD: 7.27]). The addition of GSE has likely mitigated the DDR-related adverse impact on engraftment. *IL2RG* is essential for the differentiation and function of lymphocytes, thus accounting for the profound immunodeficiency in patients with X-SCID. T cell reconstitution in transplanted mice was assessed by evaluating the percentages of circulating T cells (CD3+) as well as IL2RG expression of IL2gc. Although no significant difference in the % of CD3+ cells was observed between GE (79.77% [SD: 35.65]) and BE (91.45% [SD: 22.39]) mice, IL2gc expression in CD3+ T cells was significantly (p=<0.0001) greater in BE mice (93.85% [SD: 4.30]) compared to GE mice (59.89% [SD: 21.01]). Molecular analysis for phenotypic genetic correction of X-SCID HSPCs was assessed by digital droplet PCR for TI in GE HSPC and high throughput sequencing of target site for BE HSPCs. Mice bone marrow cells sorted for hCD45 cells revealed a significantly higher rate of mutation repair achieved by BE (84.55% [SD: 19.14]) than GE-mediated rates of TI (55.36 [SD: 18.01]). High throughput sequencing of off-targets identified by *in vitro* CHANGE-seq assay confirmed the highly specific targeting of both genome editing strategies. Base-edited X-SCID HSPCs displayed comparable engraftment and T cell development in transplanted mice compared to GE XSCID HSPCs. Of note, BE achieved significantly higher levels of genetic correction, making this a safer and more effective approach for *ex vivo* gene therapy for X-SCID patients with an *IL2RG* c.444C>T mutation.

1201 Can *PTEN-L* Gene Therapy Rescue PTEN Hamartoma Tumor Syndrome (PHTS)-Related Tumor and Behavioral Phenotypes in Mice?

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Introduction: The tumor suppressor *PTEN* is one of the most frequently mutated genes in human cancer and may cause up to 5% of autism cases. Patients with *PTEN* Hamartoma Tumor Syndrome (PHTS) have germline mutations in *PTEN* and can suffer from tumor and/or autism-related phenotypes. Female PHTS patients have about an 85% lifetime risk of breast cancer. Preventive gene therapy may improve their quality of life by decreasing the burden of frequent tumor monitoring. The longer PTEN proteoform PTEN-L is well-suited for gene therapy due to its cell-penetrating properties. Indeed, daily injections of purified PTEN-L have been shown to inhibit PTEN-null tumor growth *in vivo*. This study seeks to develop a dual-purpose adeno-associated viral vector serotype 9 (AAV9)/*PTEN-L* gene therapy for treating *PTEN*-related tumors and behavioral abnormalities. Because the endogenous PTEN signaling pathway is homeostatically regulated, one may hypothesize that unregulated *PTEN* gene therapy may cause predictable, counter-productive side effects (e.g., hyperinsulinemia) that warrant the development of regulated, second-generation gene therapies. Methods: To assess the safety of human *PTEN-L* gene transfer, male wild-type (WT) mice were injected intravenously with either vehicle or AAV9/hP599-*PTEN-L-myc* or AAV9/Cbh-*PTEN-L-myc* (1E12 vg/mouse at postnatal day P38). The hP599 promoter is a variant of the human *PTEN* promoter; CBh is a stronger, ubiquitous promoter. Blood insulin levels and peripheral PTEN-L expression were analyzed at 24 weeks post-injection. To evaluate tumor remission, female athymic nude mice (*Foxn1^{nu}*) were injected with vectors at P65-P79 and were engrafted four weeks later with PTEN-null MDA-MB-468 breast cancer cells (1E6 cells/mouse). To assess anxiety and depression, open field and tail suspension tests were conducted one month after male *PTEN$^{-/+}$* mice were injected intrathecally with either vehicle or AAV9/hP599-*PTEN-L-myc* (3E10 or 1E11 vg/mouse;

P32-P34). Results: Consistent with our hypotheses, AAV9/CBh-*PTEN-L*-myc accelerated tumor growth and caused death in about 20% of mice. In contrast, AAV9/hP599-*PTEN-L*-myc permitted 56% tumor remission by 30 days post-xenograft and did not cause any deaths. Because the hP599 vector caused weight gain and hyperinsulinemia, the development of a regulated second-generation hP599 vector may be warranted. Although not statistically significant, preliminary behavioral data trends toward normalization in the tail suspension test. Characterization of PTEN-L expression in peripheral post-mitotic tissue is ongoing. Conclusion: AAV9/hP599-*PTEN-L* demonstrated modest efficacy with anticipated side effects that may - conceptually - confound efficacy results. An improved, regulated vector design may mitigate side effects and unveil the full therapeutic potential of *PTEN-L* gene therapy.

1202 Neutrophil-Specific Single-Cell RNA-Sequencing Discovers a Novel Smad3-Dependent Immunosuppressor Prdm2 for Promoting NSCLC

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The functional role of tumor-associated neutrophils (TAN) in cancer progression is still controversial and largely unknown. Interestingly, we observed that Smad3 deficiency dramatically increase TAN population in mouse cancer model with syngeneic lung carcinoma LLC. By conducting a neutrophil-specific 10x single-cell RNA-sequencing, we uncovered the heterogeneity and transcriptomic dynamics of TAN in the Smad3-depedent tumor microenvironment (TME). Surprisingly, we found several novel TAN subsets in the Smad3-null TME which were highly distinct to the wildtype TAN. Gene ontology analysis suggested transcriptomes of the wildtype TAN were highly associated with cell cycle arrest and senescence; supporting by the prolonged lifespan of the Smad3-KO TAN in vitro and in vivo. Mechanistically, ChIP-sequencing discovered a quiescence mediator Prdm2 as a novel Smad3 direct target gene in TAN under cancer condition in vitro and in vivo, which significantly associated with NSCLC mortality. More importantly, adoptive transfer of Prdm2-silenced bone marrow derived neutrophils markedly inhibited the progression of LLC-tumor in mice. Thus, we discovered Prdm2 as a Smad3-dependent immunosuppressor of TAN, which may represent as a novel therapeutic target for cancer.

1203 Evolution of Blood-Brain Barrier Penetrant AAV Capsids in Non-Human Primates Using a Multiplexed Transcription Dependent Capsid Engineering Platform

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Many devastating diseases of the central nervous system (CNS) have the potential to be ameliorated with genomic medicines. Adeno-associated virus (AAV) is one of the most promising viral delivery modalities for gene therapy, however, transport of AAV across the blood-brain barrier poses a significant clinical challenge. Here, we employed the functional selection platform, SIFTER (Selecting *In vivo* for Transduction and Expression of RNA), to engineer capsids that transduce the CNS following intravenous administration in cynomolgus macaques. SIFTER is distinguished from other library screening strategies by the implementation of a divergent expression cassette and barcode look-up table to track capsid performance. This allows for multiplexing of different parental serotypes and diversified capsid regions in a single head-to-head experiment. For each capsid variant, multiple replicate barcodes and unique molecular identifiers (UMIs) are utilized to improve quantification of capsid performance in difficult-to-transduce tissues, including the brain. CNS-tropic capsids previously identified in a first SIFTER screening round were synthesized in a new multiplexed library of 65,000 variants comprising ten library designs across four parental AAV serotypes. In this second round of screening in cynomolgus macaques, we analyzed capsid performance in both mRNA expression and DNA delivery across the brain, spinal cord, dorsal root ganglion, and select peripheral tissues. A custom bioinformatics suite was developed to demultiplex each sub-library and compute multiple capsid performance and similarity features that describe the level and consistency of CNS transduction. The results show multiple new capsid variants with improved CNS transduction following intravenous administration relative to benchmark capsids. Blood-brain barrier penetrant capsids identified in this study will be evaluated individually for their potential utility in treating CNS disorders.

1205 Efficacy and Safety of a SOD1-Targeting Artificial miRNA Delivered by AAV9 in Mice Are Impacted by miRNA Scaffold Selection

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Toxic gain-of-function mutations in superoxide dismutase 1 (SOD1) contribute to ~2-3% of all amyotrophic lateral sclerosis (ALS) cases. Artificial microRNAs (amiRs) delivered by adeno-associated virus (AAV) have been proposed as a potential treatment option to silence SOD1 expression and mitigate disease progression. Primary microRNA (pri-miRNA) scaffolds are used in amiRs to shuttle a hairpin RNA into the endogenous miRNA pathway, but it is unclear whether different primary miRNA scaffolds impact the potency and safety profile of the expressed amiR *in vivo*. In our process to develop an AAV amiR targeting SOD1, we performed a preclinical characterization of two primary miRNA scaffolds, miR155 and miR30a, sharing the same guide strand sequence. We report that while the miR155-based vector, compared to the miR30a-based vector, leads to a higher level of the amiR and more robust suppression of SOD1 *in vitro* and *in vivo*, it also presents significantly greater risks for CNS-related toxicities *in vivo*. Despite miR30a-based vector showing relatively lower potency, it can significantly delay the development of ALS-like phenotypes in SOD1-G93A mice and increase survival in a dose-dependent manner. This data highlights the importance of scaffold selection in the pursuit of highly efficacious and safe amiRs for RNAi gene therapy.

1206 Phase I/II Trials with Sleeping Beauty Engineered CAR T Cells of Donor Origin for B-cell Acute Lymphoblastic Leukemia

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A major challenge limiting the clinical application of academic CAR T-cell immunotherapies is the manufacture of cell products using viral vectors and patient-derived material. We recently proposed the use of Sleeping Beauty (SB) transposon to engineer donor-derived CAR T cells differentiated toward the CIK population (CARCIK-CD19) for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) patients relapsed after allogeneic hematopoietic stem cell transplantation (allo-SCT). Here we report the final results of phase I/II study evaluating the safety and efficacy of CARCIK-CD19 cells in 21 patients (NCT03389035). Six additional patients were treated on a compassionate-use basis. Cells were manufactured from HLA-identical siblings, matched unrelated, and haploidentical donors. After lymphodepletion with Fludarabine and Cyclophosphamide, patients received a single infusion. Bridging therapy was allowed. A total of 27 patients (4 children and 23 adults) were infused. Successful production of CARCIK-CD19 cells was achieved for all patients. The first 9 patients were treated in dose-escalation (1x10^6/kg, 3x10^6/kg, and 7.5x10^6/kg), whereas the remaining 18 patients received 15x10^6 cells/Kg, as no dose-limiting toxicity was observed. The median number of prior therapies was 4 (range, 2-8). The median BM blast was 40% at enrolment and 1.7% after lymphodepletion. CRS occurred in 9 patients (4 grade I and 5 grade II) and ICANS (grade 3) in 2 patients at the two highest doses. Although 10 out of 27 had experienced GvHD after the previous allo-SCT, GvHD never occurred after treatment with CARCIK-CD19. Nine patients had infections, of which 6 of grade 3 or higher. CR was achieved by 18 out of 27 patients (66.7%, 95%CI=46.0-83.5%) and by 16 out of 21 patients treated with the two highest doses (76.2%, 95%CI=52.8-91.8%, 81.3% MRD-negative). With a median follow-up of 2.8 years (range, 0.05-4.4 years), at 6 months, the EFS and OS were 41.5% (SE=11.6) and 71.4% (SE=9.9), respectively. The 6-month DOR of 54.4% (SE=13.8). Robust CAR T-cell expansion was achieved in most patients and CAR T cells were measurable for up to 27 months, in association with B cell aplasia. Integration site analysis of the patient's peripheral blood demonstrated highly polyclonal marking. No signs of genotoxicity by transposon insertions could be observed. SB-engineered CAR T cells of donor origin showed an excellent safety profile with anti-leukemia activity in heavily pretreated patients with B-ALL.

1207 A Human-Centric AAV Capsid Engineering Platform for the Targeting of Dysfunctional Neural Circuits

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The fundamental cause of many neurological disorders can be attributed to the abnormal electrical activity of specific neural circuits. Normalizing activity in these dysfunctional neurons should therefore be a valuable avenue for the development of therapeutics, and ultimately the treatment of various nervous system conditions. However, for this to be a successful therapeutic approach, specific targeting is required so that anomalous neuronal activity can be corrected in the dysfunctional circuit, without altering the properties of neighbouring cells and brain regions. To advance the treatment of neural circuit disorders, Sania Therapeutics is leveraging a combination of intersectional technology platforms that allow for the targeted delivery of chemogenetic proteins through engineered AAV capsids and promoters. This approach will allow us to specifically modify neural circuit activity in disease states while minimizing side effects associated with current therapies. Directed evolution of AAV capsids has shown promise for targeting gene delivery to specific cell and tissue types, including sub-classes of neurons. However, the evolution and identification of new capsid variants has shown limited translatability across animal species and from animals to humans. Indeed, individual evolved capsids can have quite different tropisms across species making it difficult to predict how these vectors will perform when used in clinical gene therapies. Sania's R-Scan™ AAV evolution platform is designed to overcome these deficits by taking a human-first approach to capsid screening. In order to maximise the utility of our R-Scan™ platform we have generated 16 different capsid libraries based on multiple parental serotypes and diversification strategies. We have used the parental serotypes AAV1, AAV2, AAV5, AAV6, AAV8, and AAVrh10 all selected based on published studies showing useful features such as neuronal tropism, ability for retrograde transport within neurons, and low seroprevalence within the general population. To achieve a series of libraries with maximal functional variants we used a combination of random mutagenesis in the full length of the gene, mutagenesis of the external loops of the capsid, and insertions in specific locations of the capsids of either random peptide sequences or rationally designed motifs known to target our cell type of interest. Our mutagenesis libraries contain over 1 million variants each, while our semi-rational designs have around 25K variants. All these libraries were packaged in-house and characterized by PCR, qPCR, NGS sequencing, and functional testing in different cell types. Screening of these capsid libraries is carried out

in our R-Scan™ platform. This platform allows us to recreate human neural circuits in vitro and target capsid evolution to a specific neuronal sub-type. Our initial studies have focussed on the generation of human motor neuron-muscle circuits for the identification of AAV vectors that can efficiently infect human motor neurons via intramuscular injection. This in vitro circuit uses microfluidics to recreate the functional barrier between motor neuron terminals in the periphery and their cell bodies in the central nervous system. Our human motor neurons are derived in house from a pool of donor hIPSC cell lines from people of different sexes, ages and ethnicities in order to maximise translatability of these capsids across the clinical population. The R-Scan™ platform can be applied to multiple neuronal circuits and cell types and so can provide capsid variants for therapeutic uses in multiple nervous system disorders.

1208 Neutrophils Conspire with CD8+ Exhausted T Cells to Promote Breast Cancer Lung Metastasis upon Loss of BRD7

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Introduction: Thwarting metastasis is regarded as the holy grail in the treatment of cancer as metastasis accounts for more than 90% of cancer-related deaths. Intriguingly, even though epigenetic alterations have been widely implicated as a universal aspect across cancer types, little is known about how epigenetic events can engender metastasis by reprogramming the tumor immune microenvironment . Additionally, the mechanism(s) by which the loss of SWI/SNF chromatin remodeling complex subunits-like Bromodomain-7(BRD7)- induces dormant breast cancer cells to metastasize to the lung hasn't been deciphered .**Methods:** We established a novel high throughput *in vivo* screening platform which enables the identification of specific epigenetic entities that regulate metastatic reactivation. RNA-seq enabled the identification of the signaling pathways that govern metastatic dormancy. Furthermore, tumor sphere and invasion assays were performed. In order to decipher the chromatin accessibility regions regulating metastatic reactivation, ChIP-seq and ATAC-seq were conducted. A magnetic bead-based multiplex cytokine assay informed us about the regulation of cytokines. *In vivo* experiments were performed in the 4TO7-TGL and D2A1-d dormancy models and in the NSG immunodeficient mice model. Further, FACS immune-phenotyping coupled with scRNA-seq were implemented to comprehend the changes in the tumor immune microenvironment upon loss of BRD7. Finally, neutrophil and CD8+ exhausted T cell depletion was carried out by using the anti-Ly6G Ab and anti-CTLA4+anti-LAG3 Abs. **Results:** The loss-of-function screen revealed that loss of BRD7 resulted in the dormant breast cancer cells metastasizing to the lung *in vivo*. Interestingly, RNA-seq revealed that BRD7 knockout promotes the expression of genes involved in inflammation, hypoxia and EMT. Intriguingly, the top signaling pathways enriched in BRD7-silenced cells were IL6-JAK-STAT3 signaling, TNF-α signaling, and Interferon-Gamma responses. Further, ATAC-seq and ChIP-seq experiments indicated that inactivation of BRD7 caused an increased accessibility of enhancer and super-enhancer sites that were enriched for interferon-regulated response element sites. Additionally, we found that BRD7-kncokout induces the up-regulation of pre-metastatic cytokines like IL6, IL33, CXCL10, and CXCL12. scRNA-seq coupled with FACS informed us that the loss of BRD7 led to up-regulation of the tumor-promoting N2 neutrophil population and CD8+ exhausted T cells while down-regulating the tumor-suppressive M1 macrophages and dendritic cells *in vivo*. Finally, depleting neutrophils using the anti-Ly6G Ab prevented the BRD7 KO-induced lung metastasis *in vivo*. Furthermore, the efficacy of the combination of anti-LAG3+anti-CTLA4 Abs towards preventing BRD7-knockout induced metastasis by depleting the CD8+ exhausted T cells is being assessed *in vivo*. **Conclusions:** Our novel functional genomic platform shall enable the identification of specific genes that enforce tumor dormancy or mediate metastatic reactivation of breast cancer. Furthermore, our findings that BRD7 is a suppressor of breast cancer lung metastasis and a predictive cancer biomarker could have major implications in the formulation of myriad therapeutic strategies for metastatic cancers. Taken together, we anticipate that our study could potentially bring about a paradigm shift in our understanding of how epigenetic regulators, like BRD7, mechanistically regulate breast cancer metastasis and reactivation by rewiring the tumor immune microenvironment.

1209 Evolving the Components of CRISPR-SaCas9 for ATTR Gene Therapy

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Transthyretin is encoded by the *TTR* gene and acts in tetramers to transport thyroxine and retinol (vitamin A) to different tissues via blood circulation system. Instability of the TTR tetramer may lead to polymerization, forming amyloid fibrils that deposes and accumulates within tissues, such as the peripheral nerves and heart, leading to transthyretin amyloidosis (ATTR) diseases. CRISPR-Cas9-based knock-out represents a promising potentially curative treatment by permanently knocking out the disease-causing TTR. However, off-targets across the human genome and associated genotoxicity are of great concerns in *in vivo* gene therapy. To explore the potential of Cas9 nucleases beyond the most used SpCas9 for high genome targeting precision, we systematically evaluated the performance of SaCas9, a smaller Cas nuclease (1,053 vs 1,368 a.a.) with a stricter PAM (NNGRRT vs NGG), on targeting *TTR* to outline a comprehensive view on its editing efficiency and off-target effect. We screened and engineered both components of CRISPR-SaCas9 (sgRNA and *SaCas9* mRNA). We first evaluated the on- and off-target effects of all guide candidates of SaCas9 across the *TTR* exonic regions, revealing two guides outperformed over others in on-target editing efficiency and in off-target profiles. To improve the editing potency of the two candidate guides, we chemically modified parts of ribonucleotides along the sgRNA with 2′-O-methylation and phosphorothioate linkage, which significantly improved the editing efficiency by nearly 10 folds. Moreover, we optimized the *cis*-elements (5'- and 3'-untranslation regions) of *SaCas9* mRNA to further enhance its expression and thus on-target editing efficiency. Because *SaCas9* mRNA should be first translated into protein, followed by assembly with sgRNA and

then imported into the nucleus, an optimal input ratio of sgRNA to mRNA would be critical to achieve an optimal final editing efficiency. We therefore assessed a range of sgRNA-to-mRNA cargo ratios. The results showed that a ratio of 1:4 increased the editing efficiency by ~50% as compared to a ratio of 1:1 at the DNA level, as well as in TTR protein reduction. Off-target effects were analyzed by using the GUIDE-Seq method, which showed no detectable off-targets even at super-saturating dose levels at concentrations over 15 folds to the 90% effective concentrations (EC90) in any of these experiments. The editing efficiency of the optimized components of CRISPR-*SaCas9* was further verified in primary human hepatocytes. Finally, the optimized cargo encapsulated by lipid nanoparticles was delivered into rat liver through intravenous infusion and achieved a robust *TTR* knock-out in liver as compared to existing editing efficiency mediated by CRISPR-SpCas9. Taken together, we comprehensively screened and evaluated multiple attributes of CRISPR-*SaCas9* and showed great potentials of using SaCas9 on the treatment of ATTR, with noteworthy distinction on no detectable off-targets at supersaturating doses. Our data provides a reference for the systematically delivered *SaCas9* mRNA with high precision and uncompromised efficiency in *in vivo* gene editing applications.

1210 Controlling CRISPR-Cas9 Genome Editing in Human Cells Using a Small-Molecule Inducible Degradation Strategy

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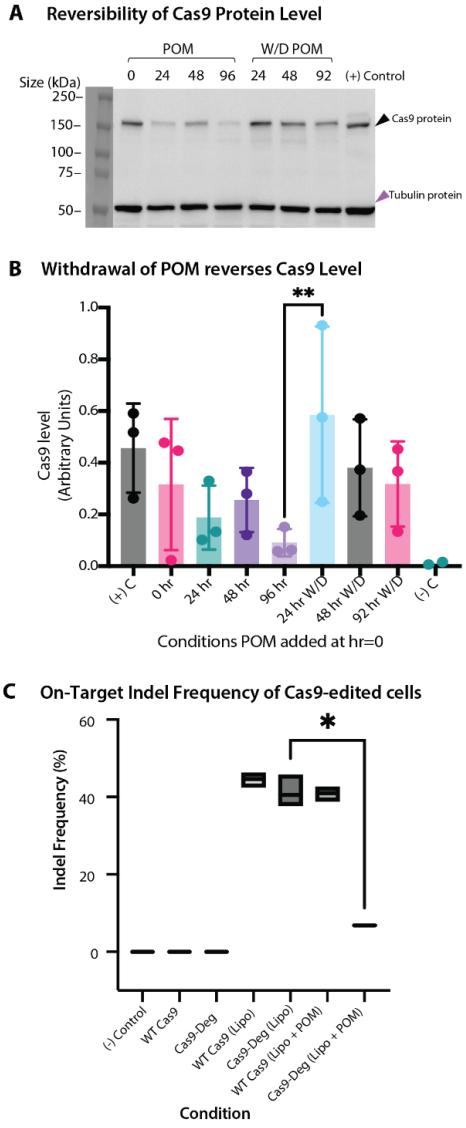
INTRODUCTION: CRISPR/Cas9 offers the advantage of targeting and cutting specific genes of interest with high precision. However, prolonged Cas9 activity has negative consequences such as off-target editing, genotoxicity, immunogenicity, and undesired on-target modifications. Current methods to control the CRISPR/Cas9 system include various small molecules, inhibitors, light and magnetic fields; however, these methods lack high-resolution temporal control over Cas9 activity *in vivo* and may leave behind residual active Cas9. Recent work has shown that a degron can be controlled by FDA-approved pomalidomide (POM). Here, we attach degrons to Cas9 and characterize the effectiveness of the degrons in degrading Cas9 and modifying the on- and off-target editing efficiency with POM. **METHODS:** Cas9-degron was inserted into human embryonic stem (H9s) and kidney (HEK293T) cells via lentiviral transduction. Varying amounts of POM were added to determine the optimal amount of drug that does not suppress cell growth or induce apoptosis. Studies were conducted to determine Cas9 protein levels once POM is added. To confirm that the degron does not hinder on-target editing efficiency, the AAVS1 Site 10 locus was targeted. DNA was extracted after two days, followed by PCR and Next Generation Sequencing. Replicates were lysed and Western Blotting was used to quantify protein. The RGEN and CRISPAltRations tool helped characterize on- and off-target editing. **RESULTS:** With the addition of POM, there is an overall decrease in Cas9-deg levels as shown by Western Blotting (Figure A). Withdrawing POM shows that Cas9 levels return within 24 hours, while a single dosage of POM can continuously degrade Cas9 protein up to 96 hours (Figure B). On-target editing efficiency (measured by indel frequency %) of wild-type (WT) Cas9 and Cas9-deg was similar (Figure C)—confirming that Cas9 activity is not hindered by the degron units. Adding POM to decrease Cas9 levels affected on-target editing efficiency significantly suggesting that Cas9-degron off-target editing activity will also decrease. **CONCLUSION:** The small molecule, POM, reversibly controls degron-engineered Cas9 protein levels as well as gene editing activity within human cells. Since, this small molecule inducer has been considered safe and is known to pass the blood-brain barrier, the system tested here has strong potential for reversible control of genome editing *in vivo* in many tissues, including the central nervous system.



A, B; Withdrawing POM reverses degradation of Cas9 protein. POM addition at hr 0 can have effects up to 96 hours and once POM is withdrawn ("W/D"), it only takes 24 hours for Cas9 levels to increase. Data represented with mean and SD, n = 3, p-values generated by mixed-effects analysis with Tukey's multiple comparisons test, against one another; ** for $p < 0.01$.

C; The degron does not hinder on-target Cas9 activity. Without a guide (No Lipo/No POM), there was no editing. Once the guide was added, both WT Cas9 and Cas9-deg had the same on-target editing efficiency. Inclusion of POM an hour before lipofecting the guide RNA shows that there is less editing for cells with Cas9-deg. Data represented with line at mean, n = 3 technical replicates per condition, p-values generated by Brown-Forsythe and Welch ANOVA tests against one another; * for $p < 0.05$.

1211 Small Volume Analytical Ultracentrifugation of AAV Vectors Based on the Band Sedimentation Velocity Method

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The accurate evaluation of particle size distribution contained in the adeno-associated virus (AAV) vector samples is one of the most important matters because the product-related impurities, including empty viral particles, and aggregates could be a possible cause of reducing the therapeutic efficacy. Based on this background, a lot of methodologies for the evaluation of particle size distribution of AAV vector were reported. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is used to quantitatively evaluate the particle size distribution in solution by observing their sedimentation behavior in the centrifugal force field. Due to the high resolution of SV-AUC with respect to particle size in solution, SV-AUC is recognized as a gold standard for the evaluation of particle size distribution of AAV vectors. Moreover, SV-AUC with a multiwavelength detection enables the comprehensive characterization of the particles contained in AAV samples[1]. However, the relatively large amount of AAV vector sample (>10^{12} particles per experiment) in SV-AUC is a matter that needs to be overcome. In this study, we report an effective approach for size distribution analysis with a small amount of AAV vectors compared to SV-AUC[2]. A small amount of AAV vectors were used in band sedimentation analytical ultracentrifugation (BS-AUC). Compared with SV-AUC, BS-AUC could be performed with about 1/50 of the AAV vector amount (4×10^{10} particles). According to the limit of quantification of this BS-AUC, the quantifiable concentrations of empty viral particles and viral vector particles of AAV8-CMV-EGFP vector were 6×10^{11} particles/mL and 4×10^{11} particles/mL, respectively. These results demonstrated that our BS-AUC approach can compensate for the drawback in terms of the sample amount of SV-AUC. The method can be also applied to other viral vectors (adenovirus vectors, retrovirus vectors, lentivirus vectors, etc.) and is very useful for characterization of them, which are difficult to manufacture in large scale. We are in the process of customizing and evaluating band forming centerpiece, to achieve higher precision and robust analysis. We plan to introduce this topic in our presentation.

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1212 Editing of a γ-Globin (*HBG1*/*HBG2*) cis-Regulatory Element in Human Hematopoietic Stem and Progenitor Cells Using Cas-CLOVER™ Technology Reactivates Fetal Hemoglobin

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Current strategies to treat genetic diseases such as β-thalassemia and sickle cell disease include delivery of a therapeutic transgene, gene correction of the inherited mutation, or γ-globin (*HBG*) reactivation to express fetal hemoglobin (HbF). Many studies focusing on gene correction or addition of a therapeutic gene require usage of viral vectors (i.e., AAV) for delivery of DNA. However, AAV has shown to be cytotoxic in human hematopoietic stem and progenitor cells (HSPCs). CRISPR-Cas9 targeting of *BCL11A*, a potent HbF silencer, can lead to inhibition of the γ- to β-globin switching process. Targeting and knockout of BCL11A raises concerns that this transcription factor may have essential roles in native erythropoiesis and/or non-erythroid contexts. Therefore, the deletion of BCL11A-binding sequences in the proximity of *HBG* could be a more ideal target instead of complete knockout of *BCL11A*. De-repression of HbF increases γ-globin expression via deletion/mutation of the binding site for the BCL11A repressor in the *HBG* promoter. Here, we aim to reactivate γ-globin expression using the high-fidelity RNA-guided endonuclease, Cas-CLOVER, to target the BCL11A binding site in the *HBG* gene promoter. Cas-CLOVER has shown high efficiency in gene editing and low off-target activity compared to the CRISPR/Cas9 system. Four pairs of guide RNAs (gRNAs) targeting the distal and proximal BCL11A binding site were designed and tested in HSPCs. Three out of four gRNA pairs showed substantial editing. For all gRNA pairs, the editing was maintained in erythroid progenitors after edited HSPCs were differentiated to the erythroid lineage. For a selected pair, we observed robust editing at Day 4, and consistent maintenance of editing at Day 11 and Day 20, respectively, during erythropoiesis. Colony forming unit assays showed that colony distribution of CFU-GEMM, CFU-GM and BFU-E were similar between mock controls and edited cells. Thus, editing using Cas-CLOVER technology does not affect HSPC multi-lineage colony forming capabilities. Finally, in vitro erythroid differentiation of edited HSPCs showed a 4 to 9-fold increase in γ-globin expression over unedited controls detected by RT-qPCR. Cas-CLOVER editing of a γ-globin/*HBG* cis-regulatory element can enhance HbF activation, which could potentially serve as an efficient therapeutic strategy for β-hemoglobinopathies.

1213 RNA Virus Based Episomal Vector for Highly Efficient Genetic Engineering of Human Mesenchymal Stromal Cells

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Introduction Although cell therapies using stem cells for the treatment of genetic disorders have been highly guaranteed to be safe in clinical studies, they often fail to demonstrate sufficient drug

efficacy. In recent years, novel ex vivo gene cell therapeutic strategies have come into the spotlight, in which the cells to be transplanted are modified in their properties by genetic engineering techniques to enhance their drug efficacy and cell proliferative potential. Therefore, there is a need for genetic engineering technology specifically for stem cell modification. We have demonstrated that the RNA virus-based episomal vector "REVec" has high transduction efficiency in mesenchymal stromal cells (MSC) and is a long-term genetic modification tool without genome integration to the host. **Methods** The transduction efficiencies of the lentiviral vector and REVec were compared by measuring immunofluorescence assay and luciferase activity after the transduction of each vector encoding GFP and luciferase into human MSCs at the same copy number. **Results** The transduction efficiency of REVec into MSCs was approximately 2-fold higher and the gene expression level in transduced MSCs was 1.5-fold higher than those of CMV promoter-driven lentiviral vector. The amount of REVec-derived transgene DNA was quantified using extracted genomic DNA of REVec-transduced MSCs and found to be below the detection limit compared to lentiviral vectors. REVec could also be transduced into bone marrow, umbilical cord blood, and adipose tissue-derived MSCs, and the gene expression level was maintained for at least three weeks after transduction in vitro. **Conclusion** Here, the novel viral vector REVec was shown to be a superior genetic transduction tool for stem cells compared to competing technologies. The high efficiency of gene transduction has significant industrial advantages, and high gene expression levels and persistence of expression will have a direct impact on the medicinal efficacy of gene cell therapies. REVec has the potential to be a standard vector for ex vivo gene cell therapeutics using genetically engineered stem cells.

1214 Evaluation of Gene Transfer Efficiency in a Mild Model of Dystrophic Muscle Disorder Performed by Machine Learning and Linear Discriminant Analysis

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Evaluation of gene therapy efficiency and the reversibility of the pathophysiological events depend on the sensitivity and specificity of the assessment to characterize the experimental model. In fact, one of the issues of translational research is the lack of prediction for some animal models. For example, multiple models of the Limb-girdle muscular dystrophy with calpain 3 deficiency (LGMD-R1) were created whereas no one can mimic the severity of the human disease. Multiple pieces of evidence showed that machine learning methods applying in image analysis could improve the efficiency and the reproducibility of phenotype evaluation. Furthermore, a supervised training method as Linear Discriminant Analysis (LDA) aimed to optimize the variability between control groups (KO-C3 and WT littermate), and to decrease the variability in each control group, by transforming the data and decreasing the number of dimensions and can be used to improve the control groups discrimination, and finally could help to increase the sensitivity to evaluate gene therapy efficiency. Here we aimed to use this approach, to evaluate a mild-phenotype rat model of LGMD-R1, and to evaluate the efficiency of gene therapy strategy. By using machine learning to first increase the reproducibility in the measurement of the histological dystrophic biomarkers, combining to functional data, and applying a supervised training method as LDA, we hypothesized that we could define a minimal efficacy dose of an AAV vector, to alleviate dystrophic phenotype in a mild phenotype KO-C3 rat model. For this purpose, at one (1) month of age, the KO-C3 rat model was injected with an AAV vector (in a dose response manner) to transfer the human calpain 3 gene into skeletal muscles. Five (5) months later, an *in vitro,* functional analysis was performed on muscle by measuring the isometric contractile properties of the muscle, and the Tetanus isometric contractions. Machine learning methods on histopathological images were used to evaluate the degeneration and regeneration of muscle fibers, the inflammation, and the fibrosis. Those data were combined with the functional data from control animals in a multidimensional dataset and transformed by LDA. After a cross-validation, and the selection of the accurate variables, the model was used to predict the efficiency of the gene transfer strategy of calpain-3. The results showed that the LDA was able to well discriminate the control animals by combining functional and histological data, in a mild-phenotype model of dystrophic disease. The efficiency of the gene transfer therapy with an AAV vector strategy was also assayed and thanks to the LDA, it was possible to predict a minimal effective dose. This study showed that the histopathological hallmarks of the disease evaluated by automatic classifier algorithm coupled to functional data and used to train a supervised LDA model allow to optimize the dystrophic features analysis and could assay the efficacy of a gene transfer strategy.

1215 Scale-Up of Suspension 293T/17-Based Cells for GMP Manufacture of AAV

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Production of AAV for early-phase clinical use requires manufacture utilizing hundreds to thousands of liters of upstream cell culture. Production facilities for early-phase trials have often relied upon adherent cell-based processes, though suspension cells offer greater flexibility and far superior scalability. We developed a transfection-based process using 293T/17 cells adapted in-house to suspension culture. Cells were cultured in 5 or 200 L bioreactors and transfected using TransIT-VirusGen. Two days post-transfection, cell lysis was performed using high-pressure homogenization and the combined media and lysate were clarified via depth filtration. Filtrate was loaded directly onto a POROS AAVX affinity column, after which additional impurities were removed via hydrophobic interaction on a Sartobind Phenyl column. After buffer exchange, anion exchange chromatography on a BIA CIM QA monolith was utilized to enrich genome-containing AAV particles. For GMP manufacture of Phase I/II material, four 200 L bioreactor runs were purified through anion exchange chromatography, buffer exchanged to PBS, formulated in 0.25% recombinant human albumin, and frozen. These four runs were subsequently combined into a pooled drug substance, diluted to a target concentration, and filled into 10 mL glass vials. Average titers across four 5 L runs were $1.74x10^{11}$vg/mL and $8.20x10^{11}$ capsids/mL, while average titers across five 200 L runs were $1.65x10^{11}$ vg/mL and $8.39x10^{11}$capsids/mL. Other

results, including cell growth and impurities, were consistent across 5 L and 200 L scales and will be presented along with several challenges related to scale-up and manufacture.

1216 A Rhabdovirus-Negative Sf-RVN® Platform is a Safer BEVS Alternative for Production of Recombinant Protein and AAV

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The Baculovirus Expression Vector System (BEVS) is a powerful eucaryotic vector system and has been used for more than thirty years as a research tool. Nowadays, the BEVS is being utilized by biopharmaceutical companies to produce recombinant adeno-associated vectors (AAV) to treat genetic diseases. The resurgence of gene therapies is providing lifesaving options to patients with otherwise untreatable diseases, leading to increased demand for large amounts of high-quality viral vectors. *Spodoptera frugiperda* (Sf) cell lines are widely used as hosts for BEVS. However, the majority of Sf9 and Sf21 cell lines contain a rhabdovirus which is considered a process contaminant and must be eliminated during the process. To improve the safety profile of the BEVS production method, we developed a high performant Sf-rhabdovirus-negative (Sf-RVN®) platform, composed of an Sf9 rhabdovirus-free cell line with an optimized chemically defined medium. Productivity of reporter protein was analyzed using the Sf-RVN® Platform as well as the production of AAV2 using the 2 baculoviruses system. Data shows that this rhabdovirus-negative platform provides increased productivity. Hence, the Sf-RVN® Platform is a performant rhabdovirus-free BEVS alternative to produce recombinant protein and AAV and enhances risk mitigation.

1217 A Novel AAV8 Capsid Variant Enabling Potent Ocular Gene Delivery in Rabbit and a Laser Induced CNV NHP Model

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Suprachoroidal space (SCS) injection is a simple and efficient technology that delivers medication materials directly into the eye, and can be done in an out-patient setting. Recombinant adeno-associated viruses (rAAVs) are emerging as the most widely used *in vivo* vehicles to deliver a targeted therapeutic gene cassette into tissues. Tropism and transduction efficiency of rAAVs greatly influence the therapeutic outcomes. The purpose of the current study is to find a new AAV capsid that can efficiently transduce retinal tissues by SCS injection. In the present study, a series of engineered vectors were generated by modifying the VP3-VIII region of a natural prototypical AAV8 capsid using a rational design method. A new capsid variant candidate v128 was selected to perform a biodistribution assay in rabbit eyes (Figure 1A-B). The transduction potency of this candidate was further evaluated in a laser-induced CNV non-human primates (NHPs) model. In a side-by-side study, the v128 candidate displayed similar tissue tropism with its prototypical AAV8 capsid vector, but has enhanced transduction efficiency for retinal pigment epithelium (RPE) and photoreceptor cells after SCS injection in rabbit eyes (Fig.1 A, B). The v128 vector has a 51.4-71.8 fold higher transduction efficiency as compared to prototype AAV8 as determined by transgene (mCherry) copy number assay (Figure 1C). As v128 candidate efficiency was determined, a further experiment is described to evaluate therapeutic potency of the candidate in a laser-induced CNV NHP model. AAV vectors carrying an anti-VEGF transgene (Aflibercept) were produced with the v128 capsid and the prototypical AAV8 capsid respectively. Each vector was injected into the SCS of the NHP eyes at a targeted dose of 1E12 vg/eye. At the evaluation timepoint, 42-days post injection, a higher concentration of transgene was found in the v128 treated NHP retina, at a level of 4056 ng/g (oculus sinister, OS) and 2112 ng/g (oculus dextrus, OD) respectively compared to prototype AAV8, and further there was considerable transgene expression also found in other ocular tissues including: aqueous humor, choroid, conjunctiva, iris/ciliary body, sclera and vitreous. In the control AAV8 group, transgene expression in NHP retina was undetectable for both eyes, and the only tissue with transgene expression was iris/ciliary body, with concentration of 39.12 ng/g (OS) and 52.8 ng/g (OD) respectively. The initial data indicates the candidate v128 capsid has demonstrated enhanced transduction efficiency in animal ocular tissues, especially the retina, compared to its prototypical capsid AAV8. The novel capsid warrants further exploration as a potential gene delivery vehicle to treat ocular disorders of the retina.

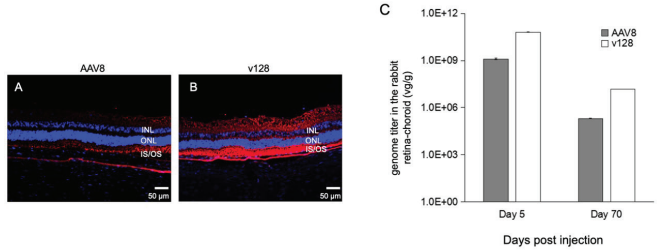

Figure 1. v128 variant shows enhanced transduction efficiency in rabbits' ocular tissues. (A-B) Immunofluorescence images of the rabbit retinal layer. 1.25E11 vg/eye of AAV8-mCherry or v128-mCherry was injected into rabbit suprachoroidal space. 21-days post injection animals were sacrificed, and the retinas were isolated. Images were taken by a confocal fluorescence microscope. (C) Genome copy numbers in AAV treated rabbits' retina-choroid tissues at day-5 and day-70.

1218 High-Throughput Arrayed Screening of Logic-Gated CARs Enables the Selection of Candidates for ccRCC with Optimal Potency and Fidelity Traits

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The development of clinically effective CAR-T cell products for solid tumors will require substantial cell engineering to confer sufficient specificity, potency, and persistence. Advances in genome engineering and synthetic biology have provided an increasingly complex set of features that can be introduced into CAR-T cells to augment their function. However, combining multiple features may result in unpredictable negative interactions between components. Here, we report the use of high-throughput screening to optimize the design of a highly-engineered Integrated Circuit T Cell (ICT) product for the treatment of clear cell renal cell carcinoma (ccRCC). ICT cells are CAR-T cells that contain an AND logic gate requiring two antigens to be present to trigger tumor cell killing together with multiple enhancement modules. First, to create the logic gate we generated hundreds of novel scFv and VH/VHH binders targeting PSMA (as a priming target) and CA9 (as a cytolytic target) via two parallel de novo binder discovery efforts: 1) transgenic mice immunizations and 2) internally-developed phage display panning campaigns. Two independent arrayed screens with 500 PSMA prime receptors (PrimeRTM) and 750 CA9 CARs were conducted to find PrimeRs with high inducibility and CARs with strong on-target potency. A fully-automated, end-to-end workflow was used to conduct each arrayed screen with the PrimeR screen evaluating PSMA binders with a fixed CAR binder and the CAR screen evaluating CA9 binders with a fixed PrimeR. Non-viral editing techniques were used to electroporate primary CD4/CD8 cells and robotic handlers were used to set up co-cultures. Lead nominations were conducted based on the desired criteria that 1) PrimeR is expressed constitutively and can induce CAR in the presence of PSMA, 2) CAR can be induced and remain stable on the surface, 3) does not elicit a response in the presence of a single-antigen and 4) CAR-dependent binding elicit strong activity that is comparable to endogenous levels of CA9.From these screens, the top 25 PSMA PrimeRs and 20 CA9 CARs were combined with an shRNA cassette for targeted knockdowns along with two variations of a persistence module. We used the same automated workflow to perform an integrated screen of the resulting 1,000 member library in T cells engineered from four human donors. Circuit specificity and potency were assessed by flow and cytokine secretion and resistance to exhaustion was assessed in a seven day killing assay. Although the library was built from components that functioned well independently, we found that when combined, many of the circuits displayed suboptimal function. Integrated screening identified 20 variants that each far exceeded the performance of a small set of initial prototypes built from top selections of individual screens. The final candidates are significantly superior to constitutive CAR-T cells in a long term killing assay, show potent cytotoxicity of low expressing antigen lines, and display background levels of cytotoxicity against single antigen targets. Engineering multiple features into T cell products is limited by unpredictable negative interactions between components. We have overcome this limitation by using high-throughput screening which generated development-ready candidates for ccRCC with finely tuned desirability criteria in <18 months.

1219 Application of ADAR-Mediated RNA Editing to Upregulate Gene Expression by Modulating Protein-Protein, Protein-RNA or RNA-RNA Interactions

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Wave Life Sciences, Cambridge, MA

AIMers—chemically modified oligonucleotides in which the chiral configurations of backbone linkages are precisely controlled at each position—direct sequence-specific adenine (A) to inosine (I)/guanine (G) RNA editing using endogenous ADAR enzymes. In addition to correcting disease-causing mutations, AIMers can be applied to modulate protein-protein, protein-RNA, and RNA-RNA interactions, thereby expanding the scope of human diseases that can potentially be addressed with this technology. We present AIMers designed to modulate the NRF2-KEAP1 protein-protein interaction system, a conserved intracellular defense pathway to counter cellular oxidative stress. NRF2 is a transcriptional regulator of antioxidant and metabolic genes that is negatively regulated through direct interaction with KEAP1, where KEAP1 sequesters NRF2 in the cytoplasm, promoting its degradation. By disrupting this interaction, AIMers activate NRF2-dependent gene expression. We designed AIMers to modify amino acids at the interface between KEAP1 and NRF2. We show AIMers direct up to 80% editing of NRF2 or KEAP1 transcripts in multiple human and mouse cell types *in vitro* and show that editing leads to dose-dependent activation of NRF2-dependent gene expression. We applied AIMers *in vivo* in mice expressing human ADAR1 and observed ≥40% transcript editing and activation of NRF2-dependent gene expression in the liver. We also present AIMers designed to alter gene expression by modifying regulatory elements in RNA that mediate protein-RNA or RNA-RNA interactions. Specific structural or sequence motifs that mediate these intermolecular interactions impact RNA processing and stability. We have designed AIMers to modify regulatory RNA elements with the aim of increasing the stability of the associated transcripts. By editing RNAs encoding multiple metabolic and immune proteins, we demonstrate that AIMers that modify regulatory RNA elements can increase mRNA expression 2-15-fold *in vitro* in cultured cells. We also show GalNAc-conjugated AIMers that lead to 40-60%

editing of these RNA regulatory elements in the mouse liver result in a corresponding ~5-fold increase in mRNA expression in liver and protein expression in serum one-week after treatment. Together, these data provide proof-of-concept that AIMers can modulate protein-protein, protein-RNA, and RNA-RNA interactions to upregulate gene expression *in vitro* and in mice.

1220 The OMNI™ Panel of Novel Engineered Nucleases Unlocks the Full Potential of Genome Editing

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Gene editing is revolutionizing molecular therapeutics. However, a key challenge in realizing the full clinical potential of this technology is the narrow repertoire of protospacer adjacent motif (PAM) sequences used by commonly applied nucleases. This restricted PAM selection limits the range of genomic targets accessible for editing and, in turn, limits the range of treatable diseases. While NGG PAM nucleases have proven useful, NGG PAM coverage across the total area of genes in the human genome is only ~5%, leaving many therapeutic relevant sites without an NGG PAM in the required editing window. Wide genome accessibility is necessary to access every potential target gene and cut site. Our solution to this challenge is based on the recognition that different nucleases utilize widely varied PAMs differing in sequence and length. With the aim of discovering novel nucleases with alternative PAM usage we have developed a dual platform technology combining a discovery pipeline and cutting-edge protein-engineering capabilities, supported by extensive computational and machine learning tools. Using this powerful platform, we have generated a panel of novel OMNI™ nucleases that unlock the full potential of genome editing. These Type II nucleases, used in our own clinical programs, are diverse in size and compatible with all delivery modalities. Importantly, our OMNI™ repertoire increases PAM usage diversity to approximately 79% gene coverage. Our engineered highly active and highly specific variants enable eliminating off-target effect and performing allele-specific editing. We are currently testing our nucleases in preclinical programs across various conditions, including severe congenital neutropenia, familial hypercholesterolemia, and retinal degenerative diseases. Our unique approach allows us to make any gene targetable, transforming the current landscape of genetic medicine.

1221 E1-Transformed BHK Cell Lines for Helper Virus-Free AAV Production Using Triple Transfection

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Adeno-associated virus (AAV) is currently the most researched viral vector for use in gene therapy. For several decades, human embryonic kidney (HEK293) cells have been the only adenovirus E1-complementing mammalian cell line suitable to produce adeno-associated viral vectors of different serotypes via triple transfection. There is a need for improved methods to address yield, scalability, and ethical concerns regarding rAAV production. To address this demand, we have developed a new cell line derived from baby hamster kidney cells (BHK-21) transformed with a fragment of the Ad5 sequence. We demonstrated this transformed BHK-21 cell line, BHK-[wt E1] stably expresses the E1 proteins. BHK-[wt E1] produced recombinant AAV by triple transfection and was confirmed based on yield, detection of viral proteins, and infectivity. FIG. 1 shows the general scheme of the experiments, and FIG. 2 shows rAAV2 viral genomes from transformed polyclonal BHK-[wt E1] cells compared to parental BHK cells untransformed (measured value below limit of detection) after triple transfection. These data demonstrate the proof of concept for this approach, and continued development and optimization of these cell lines should improve their properties. In addition, for isolating individual clones from a single cell, we used high-content imaging system to identify high-quality and pure clones for rAAV producing cell lines. The successful transformation of BHK cells as a new E1 complementing cell line and subsequent expression of high levels of E1 are important factors for rAAV production. We also discuss other strategies for creating E1-complementing cell lines using different E1 sequences. BHK-[wt E1] and other E1-complementing cell lines based on BHK may provide advantages in applications where HEK293 and similar cells are used.

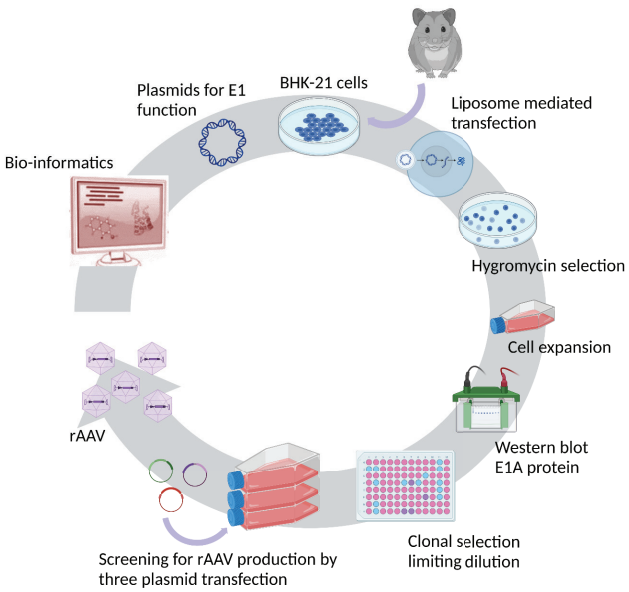

FIG. 1 Process steps for establishing and assessing BHK-21 cells for rAAV production.

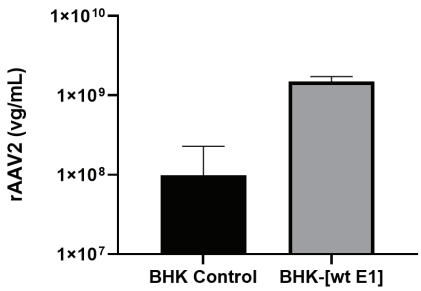

FIG. 2 Digital PCR (QIAcuity, Qiagen) measurements of rAAV2 viral genomes. BHK-[wt E1] $1.5x10^9$ +/- $2.20x10^8$, BHK Control below level of detection.

1222 Pushing the Boundaries of AAV Empty Capsid Removal: Challenges and Solutions for Resolving Half-Loaded Capsids Using Anion Exchange Chromatography

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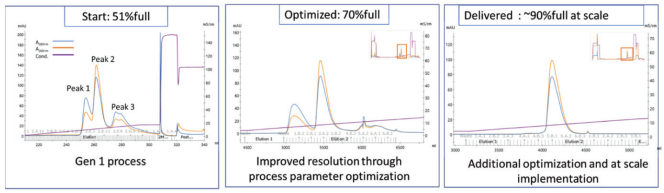
Resilience, Waltham, MA

Recombinant adeno-associated virus (rAAV) vectors have been developed as one of the most promising gene delivery candidates due to their high transduction efficacy, good safety profile, and unique tropism. However, the drug product quality impact from empty capsids remains inconclusive and requires additional downstream process capability to remove them. Though the technology of using anion-exchange chromatography (AEX) to separate empty capsids from the genome containing capsids has made significant progress in recent years, achieving good resolutions between different AAV subpopulations are still challenging due to the minute differences in their charge profiles. This becomes particularly problematic when the encapsidated gene sequence is not designed to maximize AAV load capacity. In this work, we presented a study to develop a robust and effective AEX process for an AAV product with half payload (2.3kb), where the regular AEX protocols failed to resolve the empty and genome-containing capsids. In this study, we evaluated several critical parameters that can potentially improve AEX resolution, including buffer matrices, MgCl2 concentration, column loadings, peak fractionations, etc. By combining multiple optimized parameters, the process was able to improve the %full from 50%, the original AEX process, to 90+%. Comparable %full results were reported using multiple analytical tools, including analytical ultracentrifugation(AUC), mass photometry (MP), and charge density mass spectrometry (CDMS). Full characterization of the elution profile was also conducted to understand the distribution of AAV subpopulations during AEX elution, including empty, partially-filled, full-length genome containing (2.3kb), and over-packaged (2.3+kb) capsids. The study was wrapped up with a scalability demonstration from bench-scale to pilot-scale, along with a reproducible, fully automated peak fractionation strategy for large-scale manufacturing.



1223 The Role of Natural Killer Cells During Adenovirus Infection: Implications for Adenovirus Based NK-cell Therapy

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Natural killer (NK) cells have been shown to exert a robust anti-viral and -tumor activity and were successfully applied in CAR-based cellular immunotherapy using different vectors to engineer these cells. Although there are hints in the literature that commonly used adenovirus (Ad) type 5-based vectors carrying an Ad35 or Ad37 fiber can transduce NK cells, a systemic analysis of a broad spectrum of human Ads on primary NK cells is lacking. Therefore, we focused on identifying novel Ad candidates for efficient NK cell transduction and aimed at analyzing the role of NK cells during Ad infection. To address these questions, we studied the transduction efficiencies of 21 recombinant human Ad types derived from different Ad species in peripheral mononuclear blood cells (PBMCs) and isolated NK cells derived from healthy donors. All analyzed viruses contained a dual-reporter cassette expressing green-fluorescent protein and Nano-luciferase to monitor transduction rates. We found that Ad35 from species B using CD46 as major cell entry receptor and Ad37 from species D using GD1a glycan as entry receptor revealed highest transduction efficiencies in PBMCs derived from healthy donors. To understand the role of NK cells during Ad infection, we performed co-culture experiments using primary NK cells, which were co-cultured with A549 lung cancer cells. Here, we infected A549 cells with 3, 30 and 300 virus particles per cell (VPC) for four hours with Ad35 and

Ad37, removed the virus from the culture medium and subsequently added primary NK cells for 24 hours. Quantitative PCR analysis revealed that 300 VPC showed highest transduction efficiencies in A549 cells compared to groups treated with lower VPC. Focusing on the hypothesis whether virus progeny from A549 cells results in infection of co-cultured NK cells, we performed RT-qPCR for detection of Ad-derived transgene expression in NK cells. Preliminary results revealed that NK cells were successfully infected. In the next steps, we plan to analyze the production of cytokines in NK cells in the presence of Ad infection. Furthermore, the transcriptome of these NK cells will provide more information on cell-cell interaction during Ad infection, and will enable to identify a new panel of distinct changes in NK cells upon Ad infection. We believe that this information will help to develop gene therapeutic vectors to treat orphan diseases in the future.

1224 Next-Generation Microfluidic Technology for Gene-Editing in Human Primary T Cells Using CRISPR-Cas9

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CellFE, Alameda, CA

Introduction: The exploitation of CRISPR-Cas9 ribonucleoprotein (RNP) complexes for gene editing is rapidly driving the development of non-viral gene therapies. Current trends in cell and gene therapy workflows are reliant on viral transduction to efficiently deliver genetic material. However, CRISPR-Cas9 transfected cell therapies can result in lower immunogenicity and fewer off-target effects as well as reduce manufacturing costs. CRISPR knockout (KO) is achieved through the complexation of Cas9 nucleases in combination with small single-guide (sg) RNAs to selectively target genes through site-specific recognition. For CRISPR knock-in (KI), an additional DNA donor component is included such as; single-stranded DNA, double-stranded DNA, or a plasmid, that can be added to deliver a gene of interest to the target site with limited off-target effects. We developed a workflow using microfluidic technology to effectively deliver both RNP and a DNA donor template to perform permanent genetic edits in T cells. The technology uses the unique properties of fast biomechanical deformations to rapidly edit cells while maintaining their capacity to expand and proliferate. By using the current workflow, we achieve over 15% knock-in efficiency in both Jurkat and human primary T-cells while maintaining high viability. **Materials and Methods:** In our concept validation study, we deliver CRISPR-Cas9 and donor DNA to both Jurkat cells and human primary T cells. Frozen cells are thawed two days prior to transfection, and human primary T cells are activated with the chemical activator, TransACT. At 48 hours post-activation, we use our novel microfluidic platform to co-deliver linearized double-stranded DNA with CRISPR-Cas9 RNP complexes. Our device has a high processing capacity, capable of transfecting high-cell-density samples at a rapid rate. For knock-in transfection, we process 1.5 million cells in 30 µL of native cell media through each microfluidic consumable, a process that occurs in under 1 second. T cells are directly cultured, with no need for new media exchanges, for two days, and total CRISPR events (including both knockout and knock-in results) are gathered via flow cytometry, as well as quantitative data read out through fluorescence microscopy. The cells are kept in culture for five days post-transfection to monitor the proliferation of each sample and the durability of transgene expression. **Results:** Through the homology-directed repair (HDR) pathway, we inserted an EGFP transgene into several loci; including a ubiquitously expressed target, *RAB11a*(>15% knock-in), and the T cell receptor protein, *TRAC* (>10% knock-in). Knockout data at the *TRAC* locus shows 60-80% knockout, leading to a total gene-editing efficiency of upwards of 90% using our microfluidic process. The results show minimal impact on cell health after processing, with recovery two days post-transfection (>80% viability) and no impact on proliferation. **Conclusions:** Gene therapy manufacturing is labor and time intensive, but our high processing parameters allow for rapid manufacturing. Additionally, we are able to achieve high gene editing efficiency with low cytotoxicity and low impacts on cell functionality. Our microfluidic technology represents an exciting future for non-viral gene therapy with several applications to cell therapy manufacturing.

1225 Total Lowering of HTT Using Vectorized Antisense Oligonucleotides for the Treatment of Huntington's Disease

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Huntington's disease (HD) is a fatal, monogenic neurodegenerative disease that affects between 5-17 of every 100,000 persons. Expanded trinucleotide repeats in the huntingtin gene (HTT) confer a toxic gain-of-function that causes progressive motor, cognitive and neuropsychiatric impairment for which there is currently no disease modifying treatment. Partial suppression of HTT in the brain has been demonstrated to be both safe and efficacious in animal models of HD, providing proof-of-concept for a HTT lowering therapeutic strategy. Vectorized antisense oligonucleotides (vASO) act at the transcriptional level to reduce levels of target RNA and can be expressed from adeno-associated viral (AAV) vectors for long-term target engagement. Here, we have designed and leveraged a novel vASO platform to achieve significant *in vitro* reduction of total endogenous HTT mRNA. Vectorization of ASOs involves the initial incorporation of an ASO sequence into a modified U7 snRNP (U7smOpt) sequence for nuclear localization. We first designed a novel tandem-repeat construct containing multiple copies of U7smOpt and validated this expression construct with published SOD1-targeting ASO sequences. We then generated a library of constructs expressing individual ASO sequences that collectively tiles across a target HTT exon and 100nt into its flanking introns. As these ASOs were designed to generate target knockdown by inducing exon skipping in a manner that triggers nonsense-mediated decay of the transcript, we designed and validated a novel assay for specific and sensitive multiplexed quantification of exon-skipped and total HTT transcript. The ASO library was transfected into HeLa cells that express endogenous HTT. RNA was isolated 48 hours post-transfection and assayed for exon-skipped and total HTT transcript levels. Several individual vASO sequences yielded significant target exon skipping and HTT transcript knockdown, with maximum exon skipping of 20% and maximum mRNA target knockdown of 32% relative to a control. As expected, the incidence of exon skipping events and total transcript knockdown is linked. Skipping events and transcript knockdown also appear to be caused

by ASO sequences largely targeting within the exon, with the highest efficacy seen for sequences tiling 20-30nt from the splice junctions. These results clearly demonstrate the vASO platform as a viable strategy for HTT lowering and can inform design strategies for other targets to streamline early discovery screening.

1226 Modulation of Loss of Neuromuscular Junctions in the Muscle of ALS Mice

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Amyotrophic Lateral Sclerosis (ALS) is the most common adult-onset motor neuron (MN) disease whose causes remain largely unknown and whose therapeutic options are ineffective in the long term. Hallmark of ALS is the selective and progressive degeneration of lower and upper MNs, which leads to spasticity, weakness, and muscle atrophy. Several studies suggest that non-coding RNAs are critical for ALS onset and progression. Most of studies of ALS are neuro-centric assuming that MN disfunctions are the main origin of the disease and muscle alterations are consequences. Growing evidence, however, suggest that molecular alterations in muscle fibers in the presymptomatic phase can trigger ALS pathology. However, there is still a huge gap in the knowledge about the molecular mechanisms that compromise muscle synapses and consequently MNs. To fulfil this gap, we analyzed the non-coding and coding differentially expressed genes of skeletal muscle from SOD1*G93A mice, an established model for ALS studies. We identified a signature composed by 3 miRNAs (miR-152, miR-193a, and miR-193b) that was activated in pre-symptomatic conditions of ALS and that is involved in the development, maintenance, and functionality of neuromuscular junction (NMJ). Our analysis on muscles of ALS mouse model revealed an aberrant expression of genes that encodes for acetylcholine receptors (AChRs) subunits and the results strongly suggest that the 3 miRNAs are involved in the adult-to-fetal switch of AChRs isoforms in ALS. In vitro assays confirmed that the miRNAs indeed bind the 3'UTR of target genes and effectively induce their degradation. In vivo functional assays corroborate the involvement of miR-152, miR-193a, and miR-193b in the onset of myopathies. The overexpression of the pooled miRNAs in healthy mice induced loss of muscle mass and weight reduction. The mechanisms responsible for muscle atrophy induction were also investigated. High levels of miR-152, miR-193a, and miR-193b induce the impairment of synaptic transmissions and disrupt NMJ functioning. Interestingly, we also observed the fast-to-slow myofiber shift phenomenon as a consequence of the treatment. Altogether, these results suggest that miR-152, miR-193a, and miR-193b could possibly trigger ALS, since their aberrant expression occurs in the early-stage disease. For this reason we try to modulate the expression of the three miRNAs in the muscle of ALS mouse model demonstrating that the expression of AChRs was impacted and muscle mass loss was decreased. This may represent an interesting strategy to avoid muscle atrophy and counteract muscle and motor neuronal alterations occurred in ALS patients.

1227 Development of Therapeutic Extracellular Vesicle Enveloped-AAV Vectors for Muscle Gene Therapy

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Introduction: Translating gene therapy for muscular disorders is challenging because systemic delivery of high Adeno-Associated Virus (AAV) doses is required, increasing the risks of treatment-related immune responses and adverse events. To overcome these limitations, we developed muscle-targeting extracellular vesicle enveloped-AAV vectors (myoEV-AAV), aiming to increase transduction potency to skeletal muscle and shield AAV from neutralizing antibodies (NAbs). **Methods:** We purified AAV using iodixanol (IDX) density gradient and anion exchange HPLC. We purified EV-AAV using IDX density gradient or size-exclusion chromatography (SEC). We characterized EV size, frequency, purity, and surface markers profile using NTA and western blots. We used Transmission Electron Microscopy (TEM) to determine EV-AAV structure and validate AAV packaging. We used a reporter system to determine EV-AAV transduction efficiency into HEK293 cells, myoblasts and muscle. We performed a neutralizing antibody assay (NAA) to determine EV-AAV resistance to NAbs. **Results:** Our data showed better EV-AAV purification using SEC with which we obtained one Log higher EV yield and a 5-fold higher AAV yield. SEC had better resolution in separating eluates. Using TEM, EV-AAVs had an inflated rather than a collapsed structure. EV-AAV was ~2-fold more efficient than AAV in transducing HEK293s but had similar efficiency in transducing myoblasts when we used EVs packaging myotropic AAVs (MYOAAV). Intramuscular injection of EV-MYOAAV into the tibialis anterior of C57BL/6 mice showed higher transduction efficacy than that of MYOAAVs. Using NAA, EV-AAV showed resistance to serum AAV-NAbs. Finally, we successfully targeted EV-AAVs to myoblasts using muscle-specific peptides. **Conclusion:** We have proof-of-concept for designing and producing muscle-specific EV-AAVs that transduce with high efficiency skeletal muscles and shield AAV from NAbs. Next, we will validate our results using systemic delivery of EV-AAVs.

1228 Gene Therapy for Treating Sialidosis

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Patients with sialidosis (mucolipidosis type I) typically present myoclonus, seizure, ataxia, cherry-red spot, and blindness, because of mutations in the neuraminidase I (*NEU1*) gene. Currently, there is no treatment for sialidosis. In this study, we developed an adeno-associated virus (AAV)-mediated gene therapy for a *Neu1* knockout mouse model (*Neu1*$^{-/-}$). The vector included human *NEU1* promoter, *NEU1* cDNA, IRES, and human protective protein/cathepsin A (PPCA) cDNA. Untreated *Neu1*$^{-/-}$ mice showed astrogliosis and microglia activation in the brain and lysosomal storage in dorsal rout ganglion (DRG)

neurons, together with impaired motor function. Co-expression of NEU1 and PPCA in neurons and microglia by intracerebroventricular or facial vein injection of neonatal *Neu1*$^{-/-}$ mice resulted in decreased astrocyte proliferation and microglia activation. Rotarod performance and grasping power were both improved. In facial vein-injected *Neu1*$^{-/-}$ mice, lysosomal storage in DRG neurons was decreased and body weight was improved. There was no vector-related adverse event. Therefore, AAV-mediated gene therapy is safe and efficient for treating *Neu1*$^{-/-}$ mice.

1229 Neuronal Transduction of AAV-Progranulin Corrects Microglial Lipofuscinosis and Pro-Inflammatory Microglial Morphology in a Mouse Model of Progranulin Deficiency

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State of the Art: Of the more than 70 *GRN* mutations associated with FTD, nearly all cause progranulin haploinsufficiency. Therefore, progranulin replacement is a straightforward therapeutic approach for the FTD-*GRN* patient population. There are two AAV-Progranulin gene therapeutics currently in clinical trials. PR006, manufactured by Prevail Therapeutics, is a human progranulin transgene product packaged in an AAV9 capsid which transduces both neurons and astroglia. PBFT02, manufactured by Passage Bio, is human progranulin packaged in an AAV1 capsid, which selectively transduces neurons. Our lab has developed a novel mouse progranulin construct packaged in AAV1, which despite selectively transducing neurons, also has the ability to correct microglial lysosomal dysfunction in progranulin homozygous knockout (*Grn*$^{-/-}$) mice as measured by CD68 immunostaining, and to decrease microglial soma size, as measured by IBA1-positive particle analysis of DAB micrographs. Here, we further characterized microglial phenotypes of *Grn*$^{-/-}$mice, using a combination of high resolution microscopy and computational techniques and confirmed that our novel neuron-targeting AAV-Progranulin construct has the ability to correct **both** neuronal and microglial phenotypes of progranulin deficiency.**Methodology:** A blinded experimenter used 3D Slicer to manually segment and perform 3D reconstruction of microglia from 40X Z-stacks of IBA1-stained sections of *Grn*$^{-/-}$and *Grn*$^{+/+}$ brains. We also used a MATLAB-based script which segments and skeletonizes microglia based on a threshold set by the user. We repeated these analyses with *Grn*$^{-/-}$ mice that received AAV1m*Grn* or AAV1GFP. We concomitantly measured neuronal lipofuscinosis and evaluated levels of microglial disease-associated markers in both AAV1mGrn and AAV1GFP treated mice. Finally we used confocal microscopy of IBA1-stained sections, and measured autofluorescent lipofuscin deposits within microglial ROIs, via a ImageJ/FIJI pipeline. We also developed a PYTHON-based script to quantitate the volume of lipofuscin within each microglial cell, and performed analyses to correlate microglial lipofuscin with hyperinflammatory morphology. **Results:** *Grn*$^{-/-}$ mice had age-dependent increases in microglial lipofuscinosis and microglial cell volume, territorial volume, and average branch length, and AAV1m*Grn* treatment corrected these morphologic and lysosomal phenotypes. AAV1mGrn treatment also corrected neuronal lipofuscinosis. Exogenous progranulin was detected only in neurons, not microglia. **Conclusions:** Neuronal transduction of our novel AAV-Progranulin improves the cross-correctional capacity of progranulin, allowing it to reduce both neuronal lipofuscinosis, as well as microglial lipofuscinosis and pro-inflammatory microglial morphology in *Grn*$^{-/-}$ mice.

1230 Novel Method of Producing AAV Capsid Libraries in Insect Cells

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Adeno-associated virus (AAV) capsid development via directed evolution is a powerful approach that allows relatively fast selection of virus variants with novel properties. To date, the main production platform for AAV capsid libraries used by this approach has been the HEK platform, in which capsid DNA libraries are delivered into the mammalian cells via transfection. While this method allows for the production of libraries of sufficient depth and titer, it requires the transfection of high quantities of DNA per cell, making the method prone to cross-packaging. Here, we propose a novel method for generating AAV capsid libraries in insect cells that significantly reduces the amount of DNA transfected per cell, while maintaining high AAV titers and library depth. Importantly, the capsid lead candidates selected from these libraries would be directly compatible with both mammalian and insect cell-based AAV production platforms. Our DNA libraries were built upon our proprietary plasmids, which are amplified and prepared with a three-step process to produce the AAV capsid libraries. Analysis of process intermediates revealed that even though a low amount of DNA libraries was transfected per insect cell, high AAV titers were produced, and that the efficiency of AAV recovery through the downstream process was ~40%. Furthermore, using next generation sequencing analysis we found that our method maintains the library complexity throughout the whole process, with the largest reduction in complexity occurring at the AAV production step. The level of cross-packaging of the method was measured using the limiting dilution assay followed by NGS analysis, and was found to be low compared to the cross-packaging reported for HEK-produced capsid libraries. Taken together, our data suggest that the newly proposed workflow generates AAV libraries of superior quality, suitable for AAV capsid development via directed evolution.

1231 A Novel Approach to Mass Spec Sample Preparation of AAV Capsid Protein for Peptide Mapping and Host Cell Protein Impurity Analysis

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Introduction The characterization of biotherapeutic proteins, prior to approval for use in Humans, has been the subject of immense research. Initial efforts focused hormones (Insulin), followed by the mainstream use of therapeutic antibodies. Recently, Adeno- Associated Virus (AAV) has emerged as a prominent delivery vector for Gene Therapy.

Characterization of capsid proteins and host cell protein (HCP) contaminants, has proven to pose several challenges particularly due to the stability of the viral particle and the abundance of HCPs. We have developed novel protocols for capsid peptide mapping and HCP analysis, involving precipitation of capsid proteins onto the surface of magnetic beads, followed by proteolysis with multiple proteases to maximize sequence coverage and identification of HCPs. **Results** An initial screen of Capsid solubilization revealed that heating and reducing agents were required for disruption of capsid particles, as revealed by SDS-PAGE analysis and quantitative protein assay. Surfactants improved the solubilization process, although native conditions, also gave favorable results. The use of hydrophilic magnetic beads, both as a tool for removing mass spec incompatible solutes as well as a mechanism for protein denaturation, was employed for capsid proteolysis. Our findings reveal that proteolysis with trypsin, is able to give 75 % sequence coverage of the entire VP1 Capsid protein (the longest isoform). Analysis of the Capsid sequence revealed the presence of 2 large patches of amino acid sequence (170 - 239 and 322 - 389) on the protein that predict tryptic peptides to be larger than 4000 Daltons, which is above the practical limit for peptide mapping. In an effort to increase the coverage of the protein, alternative proteases, known to be orthogonal to trypsin were utilized to improve subsequent sequence coverage. In addition, using our novel sample preparation approach, 128 human protein impurities derived from the HEK host cell were identified, with high confidence. The abundance of these host-cell impurities, relative to their natural abundance within the HEK cellular proteome will be compared. The use of additional proteases, to increase the number and confidence of protein impurities will be discussed. In addition, a DOE based approach to protocol optimization and the use of sequential digestion with orthogonal proteases will be presented. **Conclusion** We have developed an optimized, robust, and universal sample preparation method for the Peptide Mapping and Host Cell Protein Impurity analysis of AAV Capsid particles. We demonstrated that this method allows for maximum sequence coverage and identification of HCPs with high confidence.

1232 Optimizing Viral Genome Titer Methods: A Case Study on Improving Titer Accuracy Using ITR

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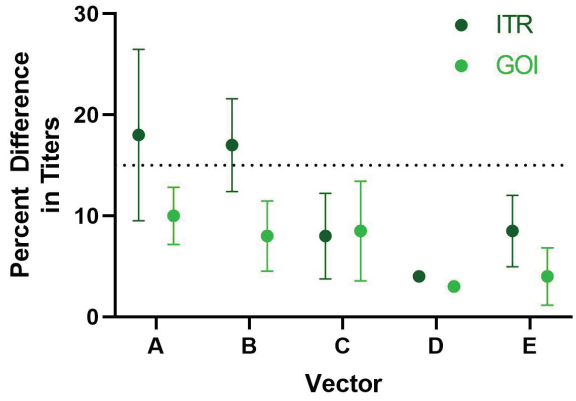
The viral genome titer method by PCR is an industry standard method to quantify AAV in order to deliver accurate doses of gene therapy products. While sequences unique to the gene of interest (GOI) are the preferred PCR target for titer determination, other targets, such as the Inverted Terminal Repeats (ITR), are desirable for early stages of development because they allow for comparability across multiple constructs with variances in GOI nucleotide sequence. The ITR can be a difficult target to work with due to its complex native structure. However, the ITR is often the only universal target when comparing constructs when down selecting lead candidates. Here we present a case study where two sample preparation methods resulted in significantly different ITR titers. This method-dependent difference was specific to vector design and only observed in two vectors out of five analyzed (Figure 1). For all vectors tested, the determined GOI titers were not significantly different between the two methods (Figure 1).



The two vectors impacted used a modified titer calculation due to differing fragmentation in the method. Fragmentation of vector DNA occurs with use of restriction enzymes that cut within the secondary structure of the ITR, such as MspI. For vectors C, D, and E, MspI cut sites are present throughout the vector genome, resulting in the ITRs being split into two different fragments. Vectors A and B have no MspI cut sites between the ITRs theoretically resulting in two ITR targets per packaged vector genome. Accurate titer quantification of Vectors A and B assumes that all vector genomes remained intact throughout the ddPCR sample analysis procedure. Fragmentation during the sample preparation was evaluated using a capillary electrophoresis (CE)-based vector genome integrity method which separates single-stranded DNA based on size. The CE data showed that heterogenous fragmentation of the vector genome occurred during the sample preparation. Less fragmentation occurred in the streamlined workflow with reduced exposure to high temperatures (>70°C). This difference in fragmentation likely accounted for the difference in ITR titers between the two methods for Vector A and B because of the unique assumption that all fragments contain both ITRs after the MspI digest. We were able to confirm this hypothesis with use of an additional restriction enzyme which splits the ITRs into multiple different fragments (Table 1). Based on these results, the additional restriction enzyme will be added to the sample preparation workflow.

Table 1. ITR differences between two sample preparation methods

	Minus Restriction Enzyme	Plus Restriction Enzyme
Method 1 Viral Genome Titer by ITR (vg/mL)	3.5E+13	2.0E+13
Method 2 Viral Genome Titer by ITR (vg/mL)	2.8E+13	1.9E+13
% Difference Method 1 vs Method 2	24%	6%

Using ITR as a PCR target allows for the use of a consistent titer method throughout the lifecycle of our programs. While the ITR may be a challenging target for PCR-based titer methods, we have demonstrated that accurate results can be achieved through sample preparation optimization. A comparison of two sample preparation workflows revealed conditions that negatively impacted the accuracy of ITR titers for a specific set of vectors. The use of an orthogonal CE method for vector genome integrity allowed the cause of the inaccuracies to be determined and an effective solution was implemented.

1233 Liver-Specific Knock-In Using Low-Dose AAV-CRISPR Restored Hemostasis in Neonatal Hemophilia B Mice with Low Antibody Response

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AAV-delivered CRISPR/Cas9 (AAV-CRISPR) has shown promising potentials in preclinical models to efficiently insert therapeutic gene sequences in somatic tissues. However, the AAV input doses required were prohibitively high and posed serious risk of toxicity. Here, we performed AAV-CRISPR mediated homology-independent knock-in at a new target site in *mAlb* 3'UTR and demonstrated that single dose of AAVs enabled long-term integration and expression of *hF9* transgene in both adult and neonatal hemophilia B mice (*mF9 -/-),* as evidenced by high levels of circulating hFIX and hemostasis restoration during entire 48-week observation period. Furthermore, we achieved hemostasis correction with a significantly lower AAV dose (2×10^9 vg/neonate and 1×10^{10} vg/adult mouse) through liver-specific gene knock-in using hyperactive $hF9^{R338L}$ variant. The plasma antibodies against Cas9 and AAV in the neonatal mice receiving low-dose AAV-CRISPR were negligible, which lent support to the development of AAV-CRISPR mediated somatic knock-in for treating inherited diseases.

1235 Clearance of Intracellular Free Cholesterol by Liver-Targeted LNP-mRNA Therapy Reverses NASH Pathology

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Cholesterol content is elevated in the livers of humans with non-alcoholic steatohepatitis (NASH) and raised levels of unesterified "free" cholesterol correlate with disease severity. High-cholesterol diets promote NASH in mouse models of diet-induced obesity. Hepatic steatosis coupled with sustained, maladaptive inflammation leads to progressive liver cell injury and fibrosis. To potentially treat this condition with high unmet need, we developed the Cholesterol Degrading Platform (CDP), an engineered fusion protein that confers on cells the ability to safely degrade excess free cholesterol into a non-toxic catabolite that is eliminated by the kidneys. Our reduction of NASH pathology with a liver-targeted lipid nanoparticle (LNP)-encapsulated CDP mRNA therapy indicates a novel approach to treating and reversing NASH. **Methods:** Male C57B6/J mice were fed a high-fat/high-cholesterol diet for 52 weeks to develop NASH. Mice were placed on normal chow, divided into two groups and treated with either (A) LNP-CDP mRNA (1mg/kg iv, qw ; n=15/group) or (B) LNP-Empty (1mg/kg iv, qw ; n=15/group) for 8 weeks. At study end, mice were sacrificed and blood and tissues were harvested and processed for analysis. **Results:** Body weights and serum AST/ALT ratios remained unchanged in LNP-CDP mRNA treated mice versus controls.

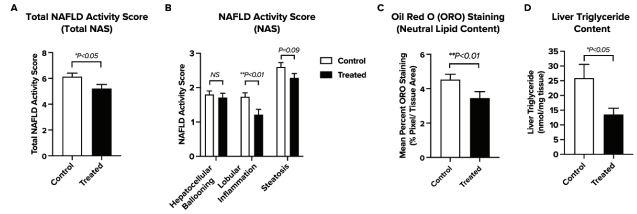

Figure 1. LNP-CDP mRNA Reduces NAFLD Activity Score in a model of NASH. A) Treatment significantly reduced total NAFLD Activity Score (NAS, *P<0.05 vs Control) which represents the sum of the scores of hepatocellular ballooning, lobular inflammation, and steatosis. B) The treatment had its greatest effects on lobular inflammation (**P<0.01 vs Control) and steatosis (P=0.09), while hepatocellular ballooning remained unchanged. While this scoring of liver steatosis did not reach our desired level of statistical confidence, treatment significantly reduced other measures of liver lipid content including C) liver Oil Red O staining(**P<0.01 vs Control) as well as D) liver triglyceride content (*P<0.05 vs Control).

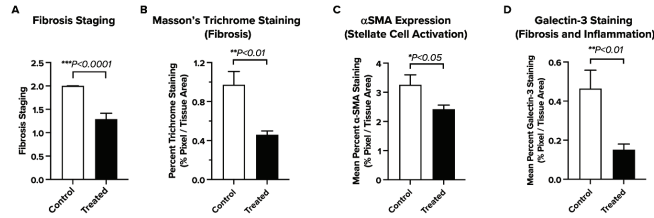

Figure 2. LNP-CDP mRNA Reduces Fibrosis and Markers of Hepatic Stellate Activation. Compared to control, treatment led to A) a significant reduction in overall fibrosis stage based on assessments of liver H&E sections (**P<0.0001 vs Control), also supported by B) a 52% decrease in Masson's Trichrome fibrosis staining (**P<0.01 vs Control). C) Treatment with REP-0002 led to a 26% reduction in α-SMA staining (P<0.05), suggesting attenuation of hepatic stellate cell activation, a major inciting mechanism and driver of liver fibrosis in NASH. Additionally, D) we observed a 67% reduction in galectin-3 staining, an established surrogate marker of tissue inflammation, fibrosis, and overall NASH severity. **Conclusion:** Our data provides strong preliminary evidence that the removal of excess free cholesterol from the liver using LNP-CDP mRNA therapy is safe and very well-tolerated, suggesting a first-in-class approach to attenuate and reverse NASH pathology.

1236 A Powerful Immunoassay Platform for Protein Expression Potency Testing

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Gene therapies offer new treatments and cures for deadly diseases, but a major challenge is the lack of fit-for-purpose analytical technologies

for monitoring critical quality attributes (CQAs). Potency is an essential CQA that has been overlooked by manufacturers and is often a sticking point with regulatory agencies. Existing analytical solutions are outdated and do not meet modern manufacturing needs. Simple Western, a capillary electrophoresis platform that incorporates a quantitative immunoassay, is a next-generation fit-for-purpose tool for gene therapy analytical testing. It excels at working in complex cell lysates or tissue homogenates, offers high sensitivity, and can monitor multiple CQAs, including potency. Here we detail a relative potency assay for a AAV gene therapy on Simple Western and characterize the analytical performance of the assay. Simple Western uses a single antibody and provides quantitative immunoassays in complex samples, making it a valuable tool in manufacturing workflows.

1237 Assessing Tissue-Specific Gene Therapies in a Pre-Clinical Mouse Model of Lipodystrophy

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Congenital generalised lipodystrophy (CGL) is a rare and life-threatening disorder. Patients with CGL fail to develop appropriate adipose tissue stores. This leads to the development of severe metabolic complications, including hepatic steatosis, lipoatrophic diabetes and cardiovascular disease. There is currently no cure for CGL, and treatment options remain limited. Adipose tissue has emerged as a target for adeno-associated virus (AAV) vectors. We recently revealed that systemic AAV-mediated gene therapy restores adipose tissue development and metabolic health in a pre-clinical mouse model of CGL. Here, we utilised the human cytomegalovirus (CMV) promoter to drive constitutive transgene expression. We have subsequently investigated whether tissue-specific AAV vectors can provide a more targeted form of therapeutic intervention for CGL. We combined AAV8 vectors with the mini/aP2 or thyroxine-binding globulin promoter to specifically target adipose tissue and liver, respectively. Additionally, AAV-mini/aP2 vectors contained the liver-specific microRNA-122 target sequence to limit hepatic expression of our transgene. Our data indicate that systemic delivery of adipose or liver specific AAV vectors restores adipose tissue development and improves metabolic health in mice with CGL. Hyperglycaemia was normalised two weeks post-treatment ($p<0.001$, n=5/AAV vector) compared to AAV-CMV-eGFP treated controls. Glucose and insulin tolerance was significantly improved, and liver weights were significantly reduced in both treatments ($p<0.0001$, n=5/AAV vector). Surprisingly, hepatic steatosis was only improved with AAV-mini/aP2 vector administration ($p<0.0001$, n=5), indicating divergent effects dependent on promoter selection. We propose that tissue-specific AAV-mediated gene therapy may offer an effective form of therapeutic intervention to correct metabolic dysfunction in patients with CGL.

1238 Unveiling the Molecular Landscape of Highly Secretory MSCs through Secretion Encoded Single-Cell Sequencing (SEC-seq)

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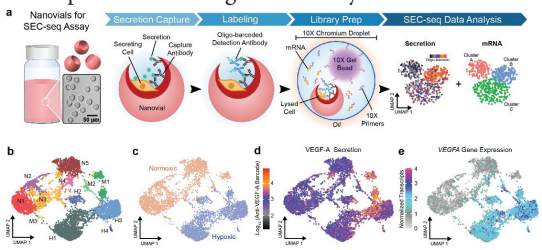
Cell function is defined by a myriad of biomolecules that they secrete. Mesenchymal stromal cells (MSCs) have been widely evaluated as therapeutics because they secrete growth and neurotrophic factors, cytokines, and extracellular vesicles, which promote immunomodulation and regeneration. However, there is a lack of methods to probe the heterogeneity in secretory functions and link these to specific gene expression networks at scale. Methods to sort therapeutic cell populations by functional potency and uncover the single-cell level gene expression driving this potency can transform the next generation of cell therapies. We introduce a new multiomics method to profile the secretion phenotype of tens of thousands of single cells and link this with the associated transcriptome using scRNA-seq. Secretion encoded single-cell sequencing (SEC-seq) leverages nanovials (hydrogel particles with nano-liter sized-cavities) and standard flow cytometry and scRNA-seq platforms to retain and analyze both transcriptome and secretion information from the same cells. Cells are loaded into gelatin-coated nanovials conjugated with capture antibodies for a secreted protein of interest, allowing the cells to adhere to the nanovial surface and secreted protein to bind to the capture antibodies. After incubation, nanovials are labeled with oligo-barcoded detection antibodies against the secreted product. Single-cell loaded nanovial samples, enriched by FACS, are then partitioned for downstream scRNA-seq, including library preparation for mRNA and oligo-barcode detection, sequencing, and data analysis (Figure 1a). We show the utility of SEC-seq by uncovering the relationship between the secretion of vascular endothelial growth factor A (VEGF-A) and the underlying transcriptome state in MSCs under normoxic and hypoxic conditions for the first time. Surprisingly, we found a relatively low correlation between VEGF-A secretion and *VEGFA* transcript levels across cells in both conditions. Yet, a subset of cells (5-20%) found in normoxic and hypoxic conditions secrete high levels of VEGF-A (Figure 1b-d; cluster M1). This subpopulation is not defined by increased *VEGFA* transcript levels; instead, it is characterized by a unique, secretory gene-enriched transcription profile. We coined this gene signature the "Vascular Regenerative Signal" due to the presence of transcripts related to cell motility, blood vessel development, and wound response, and its link to the high VEGF-A secretory state. The induction of the hypoxic response globally elevates *VEGFA* transcript levels and VEGF-A secretion across all cells, indicating that the modulation of *VEGFA* transcript levels can tune the secretion output under certain conditions. Overall, our findings demonstrate the need to probe secretion and transcriptome simultaneously to better understand

the regulation of protein secretion, and SEC-seq introduces a new method that leverages standard cytometry tools and can be broadly adopted to explore these linkages across any cells.



1239 Enhanced AaCas12b^{Max}-Mediated Disruption of a *cis*-Element in *HBG* Promoter to Generate HPFH Mutations in HSPCs for the Treatment of β-Thalassemia

Yue Meng Zhao, Yule Shao, Wenli Min, Xiaoping Zhao, Shin-Shay Tian, Su Qu

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β-thalassemia is an inherited blood disorder that is caused by a collection of *HBB* gene mutations resulting in insufficient β-hemoglobin production. Recent advances in nuclease-based approaches for genome editing, particularly applying CRISPR/Cas, have efficiently generated hereditary persistence fetal hemoglobin (HPFH)-like mutations in hematopoietic stem and progenitor cells (HSPCs) to reactivate HbF expression in erythroid lineages as an effective and endurable gene therapy for β-thalassemia. It has been demonstrated that HPFH-like mutations at the −200 bp and −115 bp upstream of the *HBG1/2* transcription start sites (TSSs) will inhibit the binding of HBG repressors and thus awaken γ-globin expression from developmental silencing. Here, we evaluated the effectiveness of a proprietary type V-B CRISPR nuclease (AaCas12b^{Max}) in combination with a single guide RNA (sgRNA) to generate genetically modified HSPCs for the treatment of β-thalassemia. Using an optimized AaCas12b^{Max}:sgRNA ribonucleoprotein (RNP) complex, we achieved editing efficiencies of >90% at the LRF binding site upstream of *HBG1/2* in both HSPCs from healthy donors and β-thalassemia patients. Indel profiles from deep sequencing showed that the optimized AaCas12b^{Max}-RNP mediated cleavage led to larger indels than spCas9 did and the most frequent mutations were >10 bp deletions by microhomology-mediated end joining (MMEJ) repairs. The AaCas12b^{Max}-RNP evaluated was highly specific for the HBG locus editing in HSPCs, with no detectable off-target activity (below 0.1% allele frequency) identified at any projected potential off-target sites as evaluated by *in silico* modeling, Tag-seq cellular assay and SITE-seq biochemical assay. To evaluate HBF reactivation after the HBG locus editing, the edited HSPCs were induced to differentiate into the erythroid lineage *in vitro*. Significant HbF induction in the edited erythroid progeny was detected by flow cytometry analysis of the F-cell (CD235a+/HbF+) percentages and HPLC analysis of the HbF protein expression. The edited HSPCs maintained their engraftment and lineage differentiation potential as evaluated together with unedited HSPCs in immunodeficient mice. The engraftment of the edited HSPC-derived erythroid cells resulted in the increase of the HbF protein levels and corrected the globin protein expression balance in the study subjects. In summary, we have demonstrated that our proprietary AaCas12bMax-RNP-mediated disruption of a *HBG cis*-element in HSPCs is a promising therapy for the treatment of β-thalassemia.

1240 Modulating Cas9 Activity and Specificity Using Antispacer PNAs for Allele Specific Gene Editing

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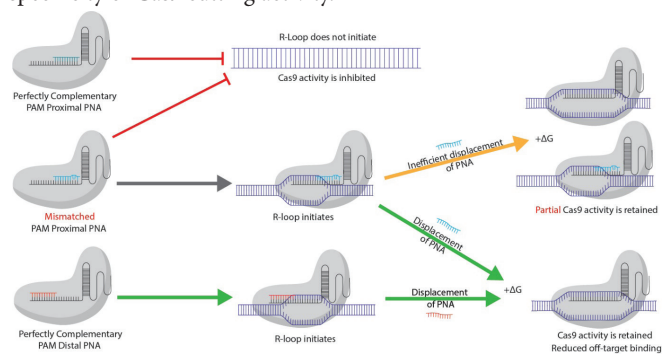
While CRISPR-Cas9 gene therapies have proven to be a powerful tool for gene editing, one area where there could be improvement is in allele-specific targeting to treat various autosomal dominant diseases in situations where preservation of the wild-type allele is required. Single base pair mismatches between wildtype and mutant sequences can prove challenging for Cas9 to differentiate depending on location relative to the PAM site. Here we use synthetic peptide nucleic acids (PNAs) that bind selected spacer sequences in the guide RNA (gRNA) to increase the specificity of Cas9. We demonstrate that PNAs which bind over the PAM distal region of the gRNA allow retention of on-target editing while reducing off-target effects. We also show that PNAs that are perfectly complementary to the PAM proximal region completely shut down Cas9 activity while PNAs with key mismatches in the same region have different abilities to modulate Cas9 editing depending on where the mismatch falls in the gRNA sequence. These effects range between complete inhibition of Cas9, moderate inhibition, or no inhibition of Cas9. We hypothesize that these distal and proximal PNA designs will allow allele specific discrimination by increasing the specificity of Cas9 cutting activity.



1241 Vector Biodistribution and Transgene Expression in the Heart Following Gene Transfer of AAVrh.10 vs. AAV9 Capsids

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AAV9 is a widely used gene transfer vector to target the heart; however, its biodistribution and functional effects relative to other cardiotropic AAV vectors, such as AAVrh.10 have not been fully elucidated. Here we investigated the differential biodistribution of AAVrh.10 versus AAV9 encoding a reporter transgene in a wildtype porcine model, as well as the functional effects of delivering a therapeutic transgene using these capsids in a mouse model of heart disease. By exploiting quantitative whole-body imaging of I-124 labelled AAV vectors, it was previously shown that AAVrh.10 distribution was ~2-fold higher in the heart when compared to AAV9 within the first 72 hours post intravenous administration in African green monkeys (Ballon et al, 2020, Hum Gene Ther 31:1237-1259). Vectors were administered intravenously to pigs, and hearts harvested for analysis two weeks post treatment. Consistent with findings in NHPs, we show using qPCR analysis of hearts from minipigs treated with AAVrh.10eGFP and AAV9eGFP, that the overall biodistribution of AAVrh.10eGFP in various cardiac regions was ~1.5-fold greater than AAV9eGFP (n=4 per vector group and n=2 for vehicle group). In addition, eGFP transgene expression analysis of porcine hearts using quantitative Westerns and immunohistochemistry also showed a similar ~1.5-fold greater expression with AAVrh.10 capsid compared to AAV9. To assess the functional impact of improved vector biodistribution and transgene expression we tested the efficacy of AAVrh.10 vs. AAV9 encoding human plakophilin-2 (PKP2) transgene in a severe mouse model of arrhythmogenic right ventricular cardiomyopathy (ARVC) harboring a mutation in the *PKP2* gene. AAVrh.10hPKP2 or AAV9hPKP2 was administered intraperitoneally at postnatal day 2 to PKP2 Hom mice (n=6 for vehicle group, n=5 per treatment group). Mice were evaluated for cardiac function using magnetic resonance imaging (MRI) 4-weeks post vector administration. MRI analysis showed improved (~2 fold) left and right ejection fraction in mice treated with AAVrh.10hPKP2 compared to mice treated with AAV9hPKP2. AAVrh.10 also showed reduced right and left end-diastolic and systolic volumes when compared to AAV9. Collectively, these data generated using different animal models demonstrate that all parameters studied (vector biodistribution, transgene expression, and cardiac functional rescue) trend better with AAVrh.10 capsid compared to AAV9.

1242 Dissecting the Mechanisms of CAR-T Cell Dysfunction in a Mouse Model of Solid Tumors

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CAR-T cell immunotherapy has demonstrated remarkable efficacy against hematologic malignancies, but their efficacy against solid tumors remains limited. Successful tumor eradication by CAR-T cells relies on maintaining highly effective antitumor T cells despite persistent antigen stimulation and immunosuppressive networks in the tumor microenvironment. CAR-T cell dysfunction has been recently explored by other groups using in vitro models of chronic antigen stimulation or CARs that induce tonic signalling. Here, we have generated a xenograft mouse model of HER2+ solid tumors in which infused anti-HER2 CD28-based CAR-T cells are driven to a dysfunctional state. We have isolated tumor infiltrating T cells at two different phases: 1) an initial phase of T cell activation that leads to tumor regression (named "effective CAR-TILs") and 2) a second phase of progressive loss of T-cell function and tumor escape (named "dysfunctional CAR-TILs"). Phenotype, function and transcriptional signatures of effective and dysfunctional TILs have been analysed. Immunohistochemistry analysis of tumors treated with anti-HER2-CAR-T cells showed CD8+ infiltration and persistence of the targeted antigen after escape to treatment. Dysfunctional TILs expressed high levels of PD-1 and had lost the expression of the proliferation marker Ki67. When using a real-time cytotoxicity assay, effective but not dysfunctional CAR-T cells were able to lyse tumor cells. We observed impaired production of cytokines and cytotoxic molecules such as IFNg, granzymes A and B and FasL in dysfunctional TILs when co-cultured with tumor cells ex vivo. However, TILs at the dysfunctional phase released higher IL-6 than effective TILs. Contrary to previous reports, the loss of effector functions observed in dysfunctional CAR-TILs was not reversible after overnight resting. This dysfunctional phenotype was not specific to CAR signalling since stimulation with PMA/Ionomycin did not rescue T cell function. Principal component analysis of RNA-sequencing data revealed strong differences between effective and dysfunctional CAR-TILs with 2.472 differentially expressed genes. Using Ingenuity Pathway Analysis, we found T cell exhaustion and senescence pathways enriched in dysfunctional CAR-TILs. We also observed NK cell-like signatures in our model as hallmark of exhaustion as recently published. Surprisingly, while effective TILs retained their effector functions and were able to induce tumor regression in mice, T cell exhaustion gene signatures were also early enriched in effective TILs. To further study the dynamics and mechanisms mediating loss of CAR-T cell function in our model we performed a soft clustering analysis of gene expression profiles from effective and dysfunctional CAR-TILs and the preinfusion product. We identified cell adhesion genes to be progressively upregulated in the dysfunctional phase, whereas genes related to metabolic and biosynthetic processes were downregulated. Using high throughput

screening we aim at providing a better understanding of the function of candidate genes in T cell effector functions and/or T cell dysfunction. Altogether our results suggest that CAR-T cell dysfunction state begins early after treatment in the tumor microenvironment and ultimately leads to treatment failure. Understanding the underlying mechanisms leading to loss of T cell functions is key to unleash the full potential of CAR-T cell therapy in solid tumors.

1243 Novel AAV9 Peptide Insertion Variants with Improved Tropism Tailored for Effective Retinal Gene Therapy

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Gene therapy has gained importance in recent years as a treatment option for inherited retinal dystrophies. In different preclinical studies, gene supplementation (or augmentation) therapy using recombinant adeno-associated viral (AAV) vectors has proven effective in halting the progression of retinal degeneration and preventing blindness. Despite many advantages, AAV vectors still have some limitations, such as the need for local delivery via subretinal injection, which carries the risk of collateral retinal damage, or limited efficiency in reaching and transducing cells outside the subretinal injection area. Here we introduce two novel engineered AAV9 variants optimized for effective photoreceptor-targeting after a less invasive intravitreal administration. These variants carry small peptide insertions in the surface exposed variable loop IV of the AAV9 capsid. AAV9 has so far not been widely used for retinal use but has certain advantages over other serotypes, such as a lower prevalence of pre-existing immunity in the human population and good neuronal tropism. Previously, heparan-sulfate proteoglycan (HSPG) binding has been shown to contribute to the retinal cell tropism of AAV vectors. Therefore, we introduced an artificial HSPG binding site with the peptide insertions to investigate how this affects penetration into the retinal cell layers after intravitreal administration. A total of ten different AAV9-based variants were assessed regarding thermal capsid stability, *in vitro* transduction properties and *in vivo* tropism in mouse retina. To this end, the novel AAV variants were packaged with a CMV-eGFP fluorescence reporter cassette and tested on several different tissue types. Transduction efficiencies were initially tested *in vitro* in HeLa cells at three different MOIs. AAV9.NNR and AAV9.GLR outperformed AAV9 wild type as well as previous AAV9 variants lacking a HSPG motif. Selected variants were also tested in *ex vivo* human retinal tissue explants with the AAV9.GLR variant showing the highest efficiency. In adult C57BL/6 mice, the AAV9.NNR and AAV9.GLR variants showed broad expression of the eGFP reporter covering the entire fundus. Immunohistochemistry at three weeks after intravitreal injection of 1E+09 total vg showed high transduction of all retinal cell layers for AAV9.NNR, while AAV9.GLR staining showed strong transduction in the photoreceptor layer. Of note, both variants also showed strong transduction of the non-pigmented epithelium of the ciliary body in C57BL/6 mice. Currently, the most promising variants are being tested in human retinal organoids to further investigate their potential for gene therapy in a complex *in vitro* model of the human retina. In summary, we describe novel promising AAV9-based vectors with improved tropism that enable less invasive and potentially safer intravitreal gene supplementation therapy for retinal diseases.

1244 Pharmacology of Clinical Candidate Lipid Nanoparticle-Mediated mRNA-Based Therapeutics for Crigler-Najjar Syndrome Type 1

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Crigler-Najjar syndrome type 1 (CN1) is an ultra-rare disorder of bilirubin metabolism caused by mutations in the uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*) gene. CN1 is primarily managed by phototherapy, which requires the individual to undergo 10-12 hours of therapy per day, impeding their quality of life. This study evaluated the pharmacology of a systemically administered clinical candidate lipid nanoparticle (LNP)-encapsulated human UGT1A1 (hUGT1A1) mRNA therapy in a *Ugt1* knockout mouse model. Newborn knockout mice were rescued from lethal postnatal hyperbilirubinemia by a single dose of LNP at 0.5 mg/kg, encapsulating the same hUGT1A1 mRNA. The rescued adult knockout mice then received a single injection of the clinical candidate LNP-encapsulated hUGT1A1 mRNA at a dose of 0.1 mg/kg, 0.5 mg/kg, or 1.0 mg/kg. Within 7 hours, serum total bilirubin levels decreased to wild-type levels (< 0.2 mg/dL) in the cohort dosed at 1.0 mg/kg, followed closely by the cohort dosed at 0.5 mg/kg in a dose-dependent manner. This reduction was sustained for 2 weeks in the highest dose cohort; subsequently, bilirubin levels rose and returned to pre-treatment hyperbilirubinemia levels by day 42 post-administration. In addition, we achieved sustained reductions in total serum bilirubin levels by repeated administration of the clinical candidate LNP-encapsulated hUGT1A1 mRNA at 1.0 mg/kg in a biweekly regimen for up to 12 weeks. Multiple doses of the LNP-encapsulated hUGT1A1 were well tolerated without any reduction in efficacy. No gross lesions were detected upon histopathological evaluation of the liver, kidney, or spleen. Findings in the liver consisted of minimal single hepatocyte necrosis (most consistent with apoptosis) with or without minimal-to-mild mononuclear inflammatory cell infiltrates. Overall, our results demonstrate that we can achieve wild-type bilirubin levels as early as 7 hours after injection of LNP-encapsulated hUGT1A1 mRNA at 1.0 mg/kg, suggesting that this may represent a potential treatment strategy for CN1.

1245 Towards Ultra Scale-Down AAV Production in Microtiter Plates

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In recent years, high-throughput screening methods have become an integral part of research and development in the biopharmaceutical industry. Due to their low cost, ease of operation and high degree of

parallelisation, microtiter plates remain an important format for early-stage process development with a strong track record of bioprocessing applications. Yet, few examples of viral vector production in microtiter plates have been published to date. This work describes the scale-down of a transient transfection-based adeno-associated virus (AAV) production process from 250 mL stirred tank reactor (STR) to 24 deep square well microtiter plates (24DSW), encompassing serotypes AAV2, AAV5, and AAV8. Initially, 24DSW material of construction and working volume were evaluated, resulting in comparable HEK293 growth kinetics to the 250 mL STR-scale. Subsequently, a Design of Experiment (DoE) approach was employed to further the understanding of the transfection step at the micro-scale. Finally, a scalable cell lysis step was demonstrated for all tested serotypes. Iterative process advances led to improved productivity, resulting in viral genome titres comparable to the 250 mL STR-scale and a reduction of the intra-plate coefficient of variance from 60% to under 30%, proving the suitability of the platform for large early-stage screening studies.

1246 Therapeutic Effects of hMSCs Administration in a Mouse Model of SCA2

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We have investigated the therapeutic potential of human mesenchymal stem cells (hMSCs), transplanted intrathecally (IT), in a transgenic mice model bearing a polyQ mutation in the ataxin-2 gene. Our results showed that IT transplantation of hMSCs at 26 weeks old could induce a significant improvement of abnormal motor function, measured by the ataxic scoring system, shown in spinocerebellar ataxia type 2 (SCA2) mice until 24 weeks after hMSCs administration. In addition, we observed that hMSCs administration protected the loss of purkinje cells through production of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), and inhibition of cerebellar inflammatory responses by production of anti-inflammatory molecules such as tumor necrosis factor stimulated gene-6 (TSG-6) and Follistatin-like 1 (FSTL1), respectively. Altogether, the administration of hMSCs improved motor behavior and ataxia-pathology alleviation by stimulating endogenous regeneration and suppressing inflammatory responses. Notably, the results of this study strongly support further exploration of the feasibility to design new clinical approaches for SCA2 patients.

1247 Natural AAV2 Variants Demonstrate Stronger Neurotropism, Penetrance of the Blood-Brain Barrier, and Differential Transduction of Brain Regions Compared to AAV9

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There is a continuing need to discover and/or engineer novel adeno-associated virus (AAV) capsids for human gene therapy. Most discovery pursuits are focused on overcoming the patient's immune system and delivering transgenes efficiently to specific tissue targets. Capsids of different serotypes possess diverse tropism profiles, resulting from variations in the amino acids present on the capsid surface. Multiple studies have used rational design to engineer capsids with desired transduction patterns. Random mutagenesis and machine learning approaches, coupled with directed evolution have enabled researchers to test large libraries of candidate capsids. These efforts have uncovered capsids that outperform those tested in clinical trials and commercially-approved AAV vectors. However, naturally circulating AAV variants remain an untapped reservoir for capsid discovery. Among a library of human-isolated 86 AAV2 variants that show equal or better packaging yields than prototypical AAV2, we identified seven variants tentatively named v46, v56, v67, v81, v224, v326, and v358, that demonstrated strong transduction following intrahippocampal injections in adult mice. In addition, we observed that the variants conferred differential transgene expression in separate structures of the hippocampus. These capsids possess 12-15 residues that are different from AAV2, mainly clustered within variable regions IV-VIII. Four of the seven capsid variants share variable region residues with AAV2.v66, a neurotropic AAV2 variant we previously identified. We have been exploring the mechanisms that underpin how these variants can confer neurotropism that rivals AAV9. Our preliminary data show that following facial vein injections of neonatal mice, the seven variants can traverse the blood-brain barrier and exhibit strong transduction of the thalamus, a structure that is an important target for neuronopathic lysosomal storage diseases but fails to be strongly transduced by AAV9 with systemic delivery. Our ongoing work explores whether the unique biodistributions achieved by our AAV2 variants are a result of cell type-specific transduction. Our findings support the notion that natural variants can serve as useful sources for gene therapy capsids.

1248 Modification of Conventional Mouse Dendritic Cells

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Dendritic cells play an important role in bridging the innate and adaptive immune response. Conventional dendritic cells (cDCs) are the most potent antigen presenting cell (APC) *in vivo*, but due to their relative scarcity and difficulty to culture, far less work has been done to optimize techniques for genetically modifying them relative to other myeloid cells, such as macrophages or monocyte-derived DCs. Here we aimed to develop and refine permanent and transient methods to modify/engineer mouse cDCs, so that they may be more effectively used in cancer immunotherapy and vaccine studies. We tested various viral transduction methods including two different adenoviral vectors (Ad5 and AdRGD) and a retroviral vector (SFG) using different promoters to improve expression. In addition, we compared and tested electroporation with DNA and mRNA using the MaxCyte ExPERT GTx. Optimized electroporation with mRNA yielded the greatest transfection efficiency (~90%) of all methods and was able to be maintained for up to 8 days, while optimized retroviral transduction yielded moderate transduction efficiency (25-50%) with long-term expression. The retroviral vector containing an additional shortened CD11c promoter had the greatest cDC1 transduction. Electroporation with DNA was able to modify cDCs (8-15%) but yielded poor cell viability. Ad5 transduced cDC1s at a low rate of ~2%, while AdRGD improved transduction by 20-fold, to 40%. These studies provide a variety of new methods for effectively engineering and modifying mouse cDCs.

1249 Systemic AAV9 Gene Therapy for Propionyl-CoA Carboxylase, Alpha Subunit (AAV9-hPCCA) in a Neonatal Lethal Model of Propionic Acidemia: Dose Finding, Biodistribution, and Long-Term Safety

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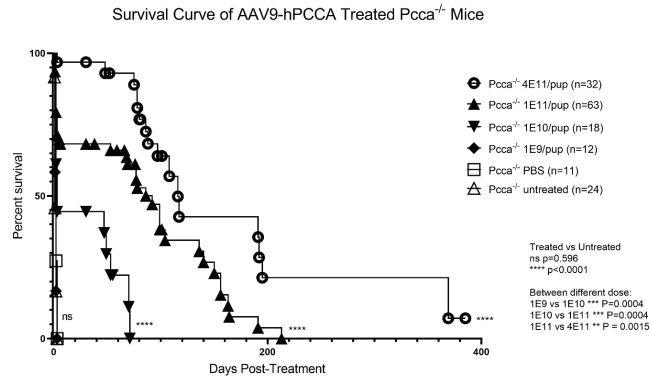
Background: Propionic acidemia (PA) is an often lethal organic acidemia caused by deficiency of the enzyme propionyl-CoA carboxylase (PCC). PCC is ubiquitously expressed and comprised of α and β subunits encoded by either the *PCCA* or *PCCB* genes. Elective liver transplantation has been offered to severely affected patients and there are no approved therapies for the treatment of PA. Systemic AAV9 gene therapy could enable hepato-cardiac correction and provide a viable alternative to liver transplantation that might also ameliorate or prevent cardiomyopathy, a devastating complication of PA. **Methods:** To develop an AAV9 gene therapy to treat PA as an NCATS sponsored Platform Vector Gene Therapy (PaVe-GT) indication, we developed and then further studied an adeno-associated virus 9 (AAV9) vector expressing the propionyl-CoA carboxylase, alpha subunit (*PCCA*) cDNA under control of the elongation factor 1 alpha (EF1a) promoter in a neonatal lethal *Pcca* knock-out ($Pcca^{-/-}$) mouse model. The most efficacious vector, AAV9-hPCCA [NCATS-BL0746], was produced at research, 10L, and 50L scales for preclinic studies to determine in vivo efficacy, explore dose ranges, and establish manufacturability. $Pcca^{-/-}$ and control littermates were treated on day of life (DOL) 1 to across the doses of 1E9, 1E10, 1E11 and 4E11 GC/pup, equivalent to ~ 7E11, 7E12, 7E13 and 2.8E14 GC/KG, respectively. In mice that were sick, aged or recently found dead, dissection and gross examination with microscopic examination in representative treated animals were performed to assess the liver, heart, kidney, skeletal muscle, and brain. **Results:** Dose-dependency was noted, with 1E10 GC/pup emerging as the lowest dose that demonstrated a survival signal (see Figure). Treatment with the AAV9-hPCCA vector was accompanied by improvements in clinical, metabolic (reduced plasma 2-methycitrate levels), and enzymatic (restoration of 1-C^{13} propionate oxidation; increased hepatic and cardiac PCC enzymatic activity) parameters. A biodistribution study using ddPCR to detect the *PCCA* transgene in AAV9-hPCCA treated $Pcca^{-/-}$ mice quantitated vector transduction in various tissues, and subsequent RT-qPCR, Western blotting, and RNA in situ hybridization confirmed robust transgene expression, particularly in hepatocytes and cardiomyocytes in $Pcca^{-/-}$ mice and $Pcca^{+/-}$ controls. In the aggregate cohort of mice treated with the AAV9-hPCCA [NCATS-BL0746] (N=283), studied over an age range of DOL 1 to DOL 765, hepatocellular carcinoma (HCC) was not detected in any $Pcca^{-/-}$ mice (N=125) or $Pcca^{+/-}$ controls (N=158). **Conclusions:** Our studies establish a range of doses that demonstrate clinical efficacy of AAV9-hPCCA [NCATS-BL0746] in a severe disease model of propionic acidemia. Importantly we document that $Pcca^{+/-}$ control littermates, some treated with AAV doses as high as 2.8E14 GC/KG on DOL 1 and observed for as long as 765 days after injection, did not develop HCC, supporting our previous hypothesis that vector design is the dominant factor predisposing mice to develop HCC after AAV integration. The AAV9-hPCCA vector used here is potent in vivo, but the AAV-hPCCA vector genome is devoid of viral and or cellular enhancers and could be readily reconfigured to express another cDNA for high dose AAV applications to achieve excellent long-term safety and no genotoxicity.



1250 Genetic Assessment of Impurity in Human iPSCs for Cell Therapy Using CE- and NGS-Based STR Analysis

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Background: The quality control of starting materials is critical for the stability and efficacy of the final products in cell-based therapy. The use of human induced pluripotent stem cells (hiPSCs) has been rapidly increased as a starting material for regenerative therapeutic intervention due to their pluripotency and large-scale manufacturing potential. One of the key concerns in hiPSC-based cell therapy is genetic stability and impurity in hiPSCs during subculture or cell banking. Recent MFDS guidelines recommend applying the Short Tandem Repeat (STR) analysis to assess the possibility of cross-contamination with other cells during the hiPSC manufacturing process, but robust method protocols remain to be verified. In this study, we established methods for genetic assessment of impurity in hiPSCs using Capillary Electrophoresis (CE)-STR and Next-generation Sequencing (NGS)-STR followed by comparative analysis of two methods with mixed-cell samples. Methods: For the assessment of impurity in hiPSCs, we used two types of stem cells; hiPSCs from the Korea National Stem Cell Bank and human induced neural stem cells (hiNSCs) from direct reprogramming of human fibroblast. The characteristic properties of hiPSC and iNSC were identified by real-time PCR and flow cytometry. To examine the sensitivity of CE-STR and NGS-STR, five mixed-cell samples were prepared using iPSCs and iNSCs from different genetic origins. Five mixed-cell samples were prepared with various ratios of 20%, 10%, 5%, 2.5%, and 1%. DNA concentration and quality of these five samples were assessed by Trio DNA Quantification Kit. CE-STR profiles were analyzed using Powerplex Kit and GeneMapper ID-X software. Precision ID GlobalFiler NGS-STR Panel v2 and Converge software v2.1 was used for NGS-STR profile analysis.Results: For the characteristic analysis of two stem cell types, we identified specific molecular marker genes in hiPSCs (OCT4 and Nanog) and hiNSCs (Pax6 and NCAD). To meet the QC criteria of genomic DNA for STR profile analysis, we confirmed that the genomic DNA from mixed-cell samples satisfied the criteria of DNA degradation index value (<1.5). When the STR profile matching percentage between mixed-cell samples and hiNSC is 56% or higher, it was defined as 'further analysis required'. In the CE-based STR method, up to 5% cell impurity (> 1:20 mixed-cell ratio) was detected, but 2.5% and 1% cell impurity (1:40 and 1:100 mixed-cell ratio, respectively) were undetected. For the NGS-STR method, we performed the QC of the library, confirming all the alignment parameters within QC satisfactory values. From the results of checking the total aligned bases using human genome hg19 as a reference, it was confirmed the sequencing was successfully performed with a value of 96% or higher. By using NGS-based STR analysis, unexpectedly, we could assess the impurity of cells when > 20% of cell impurity (> 1:5 mixed-cell ratio) existed in mixed-cell samples. The interpretation criteria are based on cell line discrimination criteria of the International Cell Line Certification Committee. Conclusion: In this study, we successfully established CE-STR and NGS-STR profiling methods which can be applied to a genetic assessment of impurity in hiPSCs using mixed-cell samples. Although NGS-STR can provide additional information on gene mutation in hiPSCs, we demonstrated that CE-STR could detect mixed cells with high detection sensitivity compared to NGS-STR. Taken together, it is necessary to choose which of two methods to apply for the assessment of impurity in starting cells according to the purpose.

1251 A Modular System to Convert Therapeutic miRNA Expression Cassettes from Ubiquitous RNA pol III-Based Promoters to RNA pol II-Driven Tissue-Specific Promoters While Maintaining Fidelity of Processing and Efficacy

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Dominant genetic diseases may benefit from RNA interference (RNAi)-based gene therapies. Although RNAi was discovered around 1998 and many RNAi-based gene therapies have been under development since that time, the approach is still emerging translationally and clinically. Many RNAi-based systems rely upon first-generation expression strategies that employ ubiquitous RNA polymerase III (pol III)-driven promoters, such as U6 or H1, to drive shRNA or miRNA expression in vivo. These systems are advantageous because they produce primary miRNA transcripts from defined transcription start and termination sites, thereby enabling consistent processing of predictable mature products through endogenous miRNA biogenesis pathways. However, these pol III-based systems do not allow cell- or tissue-specific expression. Commonly used tissue-specific promoters utilize RNA polymerase II (pol II) to drive transcription. These pol II-based systems often have multiple transcription start sites and require poly A signals and transcript poly-adenylation for termination. As a result of these sequence differences, converting a pol III-driven miRNA to a pol II-driven system can change the secondary structure of the primary miRNA transcript, thereby altering the maturation process by the RNAse enzymes Drosha and Dicer. This is important because even a single nucleotide change in a mature miRNA or shRNA sequence can impact specificity and efficiency. Thus, converting an existing pol III-based miRNA expression system to one driven by RNA pol II, and still producing the same mature miRNA, can be challenging. We previously developed and reported U6- and H1 promoter-driven systems to express therapeutic miRNAs targeting several dominant disease genes. In this study, we generated several novel miRNA designs to convert an existing U6 promoter-driven miRNA to a tissue-specific, pol II-based system. We incorporated novel secondary structure elements in the primary transcript regions flanking Drosha and Dicer processing sites, and confirmed efficacy and expression of the mature sequences in vitro. Our system will allow rapid conversion of ubiquitous to tissue-specific miRNA expression systems without rederivation of lead RNAi triggers, while also providing a new approach to restrict expression of therapeutic miRNAs.

1253 AAV Capsid Comparison in Human Neuronal Cells

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Gene therapy is emerging as a promising approach for treating a spectrum of neurological and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Adeno-associated viruses (AAVs) have been used as efficient tools for clinical gene delivery in various genetic disorders with neurological manifestations. Over a dozen AAV serotypes have been identified by natural discovery and novel capsid variants are being created by rational design and directed evolution. AAV serotypes have distinct tropisms due to their affinity to the receptors on the target tissues or cells. There are still limited platforms for evaluating AAV serotypes in human neuronal cells. Here, we establish an *in vitro* strategy to understand AAV tropism in human neuronal cells, including neuroblastoma cells and differentiated neurons derived from induced pluripotent stem cells (iPSC). We transduced the neurons and neuroblastoma cells with multiple AAV serotypes. Our results indicate that AAV serotypes have different capacities to transduce neuronal cells. These findings may contribute to AAV capsid selection in gene therapy applications to treat neuronal-related disorders.

Friday Poster Session

1254 Scalable Continuous Flow Iodixanol Gradient Ultracentrifugation Purification of Supernatant Derived rAAV8 with Enhanced Purity When Compared to Batch CsCl Gradient Ultracentrifugation

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Adeno-associated virus (AAV) is one of the most promising gene therapy vectors due to their low immunogenicity and large tropism. A robust, reliable and scalable purification process for AAV vectors is essential to the gene therapy industry. AAV purification techniques can be separated into two distinct categories, namely density gradient centrifugation or chromatography purification. For the serotype-independent purification of AAV vectors produced in HEK-293 or SF9 cells, the two most widely established methods for preclinical applications are based on batch ultracentrifugation using either cesium chloride (CsCl) or an Iodixanol density gradient. Importantly, Iodixanol is inert and non-toxic to mammalian cells, and should be favored for scaling up. In addition, Iodixanol has sufficient viscosity to form a stable gradient. Reported data suggest that vectors based on many commonly used AAV serotypes are present at high titer in culture medium of transfected cultures, and can be recovered simply by harvesting and concentrating the culture fluids. Here, we report a simplified and scalable rAAV8 vector manufacturing process using supernatants of triply transfected HEK 293 cultures and PEG precipitation strategy. To this end, we compare the final product profiles obtained : 1) via 2 rounds of CsCl gradient-based batch ultracentrifugation and 2) 1 round of Iodixanol gradient-based continuous flow zonal ultracentrifugation. Data obtained shows the presence of a significant quantity of rAAV8 viral particles in the supernatants of the cell cultures. This quantity is similar to one that can be obtained after cell lysis. A significant improvement of impurities profile (decrease of residual total host cell protein and cellular DNA) in the final product has been observed after purification through Iodixanol gradient performed using continuous flow ultracentrifugation with respect to the one obtained after cesium chloride gradient batch ultracentrifugation while total particles recovery (process efficiency) and vector purity are similar in both purification processes. Due to its high capacity and scalability these data suggest that supernatant derived rAAV purified with one round of Iodixanol gradient-based ultracentrifugation could be a suitable process for producing enough material for clinical application in cost and time effective way.

1255 The Impacts of Suppressing Cellular Innate Immune Responses on Adeno Associated Virus Yields During Production

Paul Kroeger[1], Khan Umaer[1], Ngan Trinh[1], Andrea Vervoort[2], Srivanya Tummala[1], Ben McLeod[2], Brian Tomkowicz[1]

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Cellular immune response to viral infection, as well as neutralization due to pre-existing antibodies, are significant clinical hurdles to overcome for successful viral vector mediated therapies. However, very little attention has been placed on understanding how the cellular innate immune response impacts recombinant AAV production during manufacturing. It has been recently reported that during the triple transfection process, a common technique employed for recombinant AAV generation, indeed, a multi-phasic innate response, accentuated by upregulation of inflammatory, antiviral, and interferon-stimulated cytokines genes and proteins is prominent in HEK293 production cells. In a collaboration between Virica Biotech and the Center for Breakthrough Medicines, we sought to better understand how suppressing the cellular innate response would impact virus production using Viral Sensitizers (VSE™). VSEs are proprietary small molecules that are designed to target cellular immune responses with the overall outcome of improving viral yields and effectiveness. VSEs accomplish this by transiently altering cell signaling to improve yields. Virica Biotech has a library of hundreds of VSEs each of which attenuate the antiviral defense in different ways. We screened eight compounds representing unique classes of antiviral defenses and identified several VSEs that increase overall virus and full particle production, as measured by dPCR & ELISA, in small-scale, 30mL screens. Identification of leads that showed optimal performance were then used in shake flask studies to examine the impact these compounds have on the distribution of full particles following purification using a two-step AKTA-based affinity and anion-exchange polish. Our results

demonstrate that the addition of viral sensitizers skews particle yields to a "full" phenotype, in agreement with published observations that cellular innate immune responses impact virus production.

1256 Evaluation of the Manufacturability of MyoAAV Capsid Variants

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Directed evolution of adeno-associated virus (AAV) capsids has the potential to drive exciting breakthroughs for tissue-specific gene therapies. The advantages of these novel capsids are in their ability to target specific cell types with high efficiency resulting in a significantly lower dose required to elicit a therapeutic benefit compared to natural capsids. However, these same capsid surface modifications that alter tropism and potency may also affect the manufacturability of the vector, i.e., impact productivity, purification, and stability. The incompatibility of novel capsids with an established manufacturing platform can result in extended process development timelines, thereby delaying access of transformative therapeutics to patients. Therefore, early determination of manufacturability can either de-risk a development candidate or aid resource allocation planning. Moreover, although biodistribution and biological activity will ultimately define the selection of a novel capsid, performing a manufacturability assessment as part of a comprehensive capsid engineering and screening exercise can aid in the selection of an ideal capsid variant for clinical development. The Directed Evolution of AAV capsid Leveraging In Vivo Expression of transgene RNA (DELIVER) platform is a methodology to select highly potent AAV capsid variants with ability to transduce specific cell types through the application of directed evolution and machine learning. The DELIVER methodology begins with a library of >5,000,000 capsid variants in the first round of selection; yielding approximately 30,000-60,000 variants introduced in the second round; and resulting in the identification of 10-30 primary capsid variants. These primary capsid variants are evaluated for manufacturability in parallel to the final round of screening. We have implemented a manufacturability strategy that applies metrics for assessing productivity in suspension culture, compatibility with affinity-based purification processes, and consistent product quality attributes. The productivity of candidate capsid variants was determined using a scale-down model of a suspension-adapted HEK293 transient transfection process. Cultures were harvested by chemical lysis, clarified by centrifugation, and processed by affinity chromatography. All process parameters were held constant for each capsid variant for the preliminary assessment. Samples post-lysis, post-clarification, and post-chromatography were analyzed by ddPCR to determine vector titer. Multiple MyoAAV capsid variants were tested within the manufacturability paradigm. All variants demonstrated similar productivity in cell culture harvest at quantities sufficient to support clinical development. Further, all variants bound and eluted from an AAV9 affinity column using standard conditions. Each variant also demonstrated expected quality attributes for AAVs (e.g. capsid protein ratio, capsid size, etc.). The totality of the data generated during the assessment showed similar manufacturability for all MyoAAV variants tested - suggesting that insertion of peptide sequences in variable region VIII does not significantly alter the productivity in HEK293 cells, nor impact vector quality.

1257 Bench Scale Process Development of RNA-LNPS Using a Novel Microfluidics Platform

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Introduction: The RNA-lipid nanoparticle (LNP) vaccines for the SARS-CoV-2 pandemic highlight the impact of genetic medicines deployed at scale. RNA-LNP applications also include gene editing, oncology, and rare diseases. However, a common need for these RNA-LNP therapies is a scalable manufacturing process. The mixing process to encapsulate RNA within LNPs is among the most difficult unit operations to scale-up throughput rates and batch sizes. In this work, we demonstrate the capabilities of a novel microfluidic-based instrument that enables bench scale production of 60 mL of pre-dilution RNA-LNPs at flow rates of 200 mL/min. These capabilities allow rapid and cost-effective process development with the same critical process parameters (CPPs) and downstream unit operations that are used for large-scale cGMP production. **Methods:** Self-amplifying mRNA (saRNA) encoding the SARS-CoV-2 spike protein and a custom lipid composition were used as a model for a RNA-LNP vaccine. The total flow rate and formulation volume was increased stepwise from 12 mL/min and 5 mL to 200 mL/min and 40 mL using the NanoAssemblr Ignite+ instrument with the standard NxGen microfluidic mixer or larger NxGen 500 mixer. Post-formulation dilution ratio, N/P ratio, and flow rate ratio were kept constant. The saRNA-LNPs were purified with ultrafiltration or TFF. Size, polydispersity and zeta potential were measured by DLS, and RNA encapsulation efficiency by Ribogreen assay. The lipid composition of the saRNA-LNPs was assayed with UHPLC. The saRNA integrity was assayed through capillary gel electrophoresis. *In vitro* expression was assessed in BHK570 cells. Immunization was done by intramuscular injection, with a booster on day 28. Tail bleeds were 1 day before each immunization and mice were culled on day 42. IgG titers were determined by ELISA. **Results:** All the saRNA-LNPs were of high quality with particle sizes ranging from 75 - 87 nm, polydispersity indices < 0.16 and RNA encapsulation efficiency > 95%. *In vitro* spike protein expression was similar across all process conditions with EC50 values between 0.06 to 0.12 μg/mL. Immunization of mice with the saRNA-LNPs resulted in an IgG specific response between $1.4x10^4$ to $1.8x10^5$ ng/mL. Additionally, the 40 mL batches enabled downstream processing by TFF. Finally, the saRNA-LNPs were similar to those produced by larger clinical-scale instruments. **Conclusions:** We have developed a microfluidic instrument and workflow for enabling bench scale process development of RNA-LNP drugs with the same CPPs used for GMP production. Sufficient quantities of these RNA-LNPs can be produced for optimizing downstream unit operations such as in-line dilution and TFF, and for large cohort animal studies, stability testing and extensive analytical

characterization. Overall, this technology should enable researchers to begin process development activities much earlier in drug development, thereby decreasing cost, risk, and timelines.

1258 Automated Systems Leading Solution to Increase LVV Characterization Capacity

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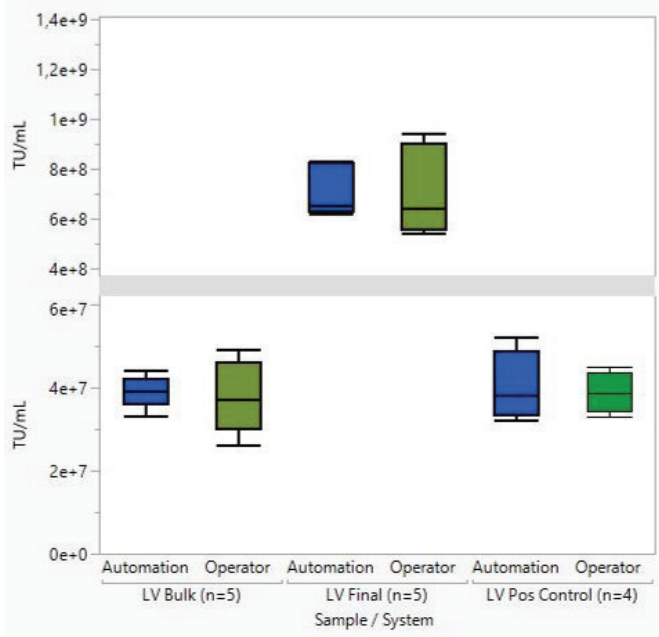
AGC biologics is a leading CDMO, providing world-class development and manufacture of therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells. Lentiviral vectors (LVV) are efficient vehicles for gene delivery that play an important role for ATMPs. The efficiency of the production process and the performance of the final LVV is monitored through potency and purity methods. In AGC Biologics, the infectious and physical viral titers and the ELISA assays to determine residual Host Cell Proteins, total residual DNA and residual Benzonase have been automated to increase capacity while also allowing the flexibility to run different assays in parallel using the same sample aliquot. The automated stations are constituted of a core liquid handling, full integrated with all the devices, Sealers, Real Time PCR thermocyclers, plate wash and plate reader, incubators to correct perform the different steps of the assays, including standards, controls and samples dilutions, procedure steps, results analysis. The methods have been validated according before being transferred into automation and comparability of each assay's automated and manual methods has been demonstrated, with p values ranging from 0.800 to 0.090, repeatability ≤ 20% and a reproducibility with manual execution system ≤ 19%. Among the assays mentioned above, the infectious viral titer method is the most complex and time-consuming method, where the automation has brought the greatest help in terms of number of samples analysed at the same time. The assay is based on the detection of proviral DNA copy number integrated into the cell genome. The selected reference cell line, deeply characterized and full representative of target primary cells, is transduced with serial dilutions of vectors and tested with a qPCR assay. Regarding infectious titer, the automated solutions brought to 5-fold increase in number of samples daily tested, greatly reducing the timing of analysis and operator effort. Results generated with automatic stations on different purification steps or final purified LVV are comparable to the manual ones, Figure 1, with a repeatability ≤ 15% and a reproducibility with manual execution system ≤ 19%. All the automated stations, compliance to CFR21 and qualified according to ICH Q2 (R1), allow to have a complete automatic and traceable flow, from sample vial to result, that makes the difference in laboratory capacity. **Figure 1**. Representative data obtained from the comparability study. Reference cell line has been transduced with LV bulk (n=5), LV final product (n=5) or LV positive control of transduction (n=4) in independent experiments. The same samples have been processed either with automation or with the validated method. Results of the same sample have been analysed with pooled t-test assuming equal variance. The two data sets are not significantly different for all the samples tested (p ≥ 0.787).



1259 Engineering CRISPR-Based Editors for Treatment of Machado-Joseph Disease

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Machado-Joseph Disease (MJD) is a dominantly inherited neurodegenerative disorder, integrating the group of polyglutamine (polyQ) diseases as the most prevalent form of spinocerebellar ataxia. MJD is caused by the overexpansion of a cytosine-adenine-guanosine (CAG) trinucleotide repeat in the *ATXN3* gene encoding the Ataxin-3 deubiquitinase. As a result, the formation of an expanded polyQ segment within Ataxin-3 confers a toxic gain-of function to this mutant isoform manifested by the generation of protein microaggregates and toxic species that trigger neuronal cell death by disrupting vital cellular mechanisms. Suppression of *ATXN3* expression by RNA interference sequences is the most widely adopted approach to treat MJD. However, the role of Ataxin-3 in numerous cellular functions may be indicative that fully silencing its activity could be deleterious to patients. Here, we harnessed the ability of CRISPR-base editing technology to target the *ATXN3* genomic locus and precisely insert A>G or C>T point mutations to permanently remove the MJD-causing polyQ segment from the Ataxin-3 while keeping its physiological activity. For this purpose, we designed CRISPR-base editor sequences that (i) modulate

ATXN3 splicing to exclude the CAG-encoding exon 10 by disrupting conserved nucleotides within splicing sites, or (ii) introduce nonsense mutations to create premature stop codons in the exon 9 upstream of the CAG tract. We show that CRISPR-base editors can precisely insert single point mutations in the *ATXN3* locus with up to 70% efficiency, both in HEK293T cells or patient-derived induced pluripotent stem cells (iPSC). This results in the formation of a C-terminal truncated Ataxin-3 isoform lacking the polyQ tract previously reported to retain its ubiquitin-binding and deubiquitase activity. *ATXN3*-targeting base editing sequences were successfully packaged into a dual adeno-associated virus (AAV) vectors that efficiently reconstitute the CRISPR editor framework by intein-mediated protein trans-splicing. The capacity of CRISPR-base editors to edit *ATXN3* and rescue MJD neurodegeneration is currently being validated in patient iPSC-derived neuronal cultures and a transgenic mouse model. Our work illustrates the potential of CRISPR-base editors as a novel and safe gene therapy approach that could positively impact the life quality of MJD patients.

1260 High-Resolution Genetic Stability Analysis in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Cell Therapy

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Background: The use of human-induced pluripotent stem cell (hiPSC) as a starting material for cell therapy has been increased due to its pluripotency and large scale-manufacturing potential. However, the genetic stability of hiPSCs and their differentiated target cells have to verify since certain genetic variants could be associated with an increased risk of tumorigenicity or dysfunction for regenerative therapeutic intervention. In this study, we assessed the genetic stability of hiPSCs and hiPSC-derived cardiomyocytes (CMs) during the proliferation and differentiation process using next generation sequencing (NGS)-based analysis. Methods: To examine the genetic stability of hiPSCs or hiPSC-CMs, we performed CytoscanHD array and NGS-based analysis such as whole exome sequencing (WES), targeted (tumor) panel sequencing (TS) at 0, 2, and 4 weeks after differentiation of hiPSCs into hiPSC-CMs. For the prediction of tumorigenicity from genetic mutations in hiPSCs and hiPSC-CMs, we analyzed the mutation data using COSMIC (Catalogue of Somatic Mutation in Cancer), reflecting a high risk of cancer mutation. We further analyzed their functional and structural pathogenicity using multiple bioinformatics tools including Mutation Taster software. Results: First, we detected a total of 25 sub-chromosomal copy number variations (CNVs) with sizes ranging from 7.3 kb to 2.5 Mb including 3 deletions and 22 gains in the process of sub-culture of hiPSCs and differentiation into hiPSC-CMs by NGS-based analysis. On chromosome 20 q11.21, copy number gain was detected with the size of 2.5 Mb CNV which was not detected by conventional karyotyping. Next, using WES analysis, we detected 425 of single base pair mutations which were found only in hiPSC-CMs at 2 and 4 weeks of differentiation from hiPSCs. Among those variants, three mutations were verified as a high risk of tumorigenicity with nonsense mutation or frameshift mutation. MUC4 c.8032_8033insA; p. (Pro2678fs) and KMT2C c.2263C>T; p. (Gln755Ter) were analyzed to be associated with potential tumorigenicity. AKAP9 c.4004_4006dupAA; p. (Lys1335_leu1336insGln) could be associated with potential abnormal myocardium. From the results of targeted (tumor) panel sequencing, we detected additional two genetic mutations such as BCOR c.1487_1500del; p. (Ile496AsnfsTer17) and BCOR c.2457_2460del; p. (819AsnTer) that could not be found by WES due to read depth differences. Conclusion: In this study, we demonstrated that the tumorigenicity-related genetic variants could be detected during the differentiation of hiPSCs into hiPSC-CMs by using various NGS-based analyses. The high-risk tumorigenic mutations were detected by only high-resolution analysis such as WES and TS, but not by conventional karyotyping. These results suggest that a high-resolution genetic analysis followed by an orthogonal validation method is not optional for assessing the genetic stability of hiPSC and hiPSC-derived target cells for cell therapies.

1261 Restoration of Brain Cholesterol Metabolism as Gene Therapy Strategy in Huntington's Disease: Unilateral Injections Are Insufficient to Elicit a Treatment Effect in HD Mice

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Impaired brain cholesterol metabolism is commonly identified in severe neurodegenerative disorders. In Huntington's disease (HD), impaired brain cholesterol homeostasis is caused by mutant Huntingtin (mHTT) deposition and is a major driver of HD hallmarks. Cholesterol 24-hydroxylase (CYP46A1), a key neuronal enzyme of cholesterol metabolism, is reduced in HD patients' brains. CYP46A1 removes the excess of cholesterol from neurons, thus regulating cholesterol synthesis and content in cell compartments. CYP46A1 is also is a key neuronal stress response factor in pathological conditions. In HD, reduced CYP46A1 is associated with extensive cholesterol accumulation in neuronal membranes with multiple consequences: impaired vesicular transport and synaptic transmission, compromised clearance of misfolded mHTT and compromised energy metabolism. Preclinical studies have indicated that restoring CYP46A1 levels and thus re-establishing cholesterol metabolism in striatal neurons by mean of an Adeno-Associated Virus (AAV) vector, considerably alleviated neuronal dysfunction and lowered mHTT accumulation through improved autophagy (Boussicaut et al. 2016; Kacher et al. 2019). In this study, we aimed at comparing the outcome of unilateral vs bilateral administration of AB-1001 (AAVrh10-CYP46A1) on motor and neuropathological outcomes in order to support, unilateral vs bilateral administration of AB-1001 in HD patients. The general locomotor activity (openfield) demonstrated no significant differences between R6/2 mice injected unilaterally or bilaterally with AB-1001 relatively to control R6/2 mice. The cylinder test revealed no differences among R6/2 mice injected with AB-1001 unilaterally or bilaterally, compared to R6/2 mice, also indicating the absence of forelimb asymmetry in mice

injected unilaterally. However, motor coordination analysis assessed by rotarod, showed that AB-1001, when administered unilaterally in R6/2 striatum up to titer 1.1E9 vg/µL (highest dose concentration in the human trial), did not improve motor performance of R6/2 mice. Bilateral intra-striatal AB-1001 delivery confirmed the beneficial effects of AB-1001 found in previous dose-response studies: motor performance of R6/2 mice injected bilaterally with AB-1001 at lower dose concentration (0.6E9 vg/µL) was improved by 60%.Interestingly, in R6/2 mice injected unilaterally with AB-1001, a significant reduction of aggregate number was observed [titers 0.6E9 vg/µL (25%) and 1.1E9 vg/µL (28%)] in injected striatum, accompanied with slight non-significant decrease (15-17%) in the contralateral non-injected hemisphere, despite the absence of vector genomes observed in that hemisphere. In R6/2 mice injected bilaterally with AB-1001 at 0.6E9 vg/µL, a comparable decrease on the number of aggregates (28%) was observed in both hemispheres. Neurofilament light-chain (NfL) levels (marker of neuro-axonal damage) remained unchanged in R6/2 mice injected unilaterally with AB-1001 at 0.6E9 vg/µL while a decreased trend was observed in mice injected unilaterally with the highest titer of AB-1001 (1.1E9 vg/µL; no motor benefits) and in R6/2 mice injected bilaterally with AB-1001 injected at lower titer (0.6E9 vg/µL), compared to control R6/2 mice. There were no adverse effects after unilateral/bilateral dosing of R6/2 mice with AB-1001. These data support that bilateral injections are beneficial for obtaining a clinical therapeutic effect of AB-1001 and that unilateral injections are insufficient in eliciting a treatment effect

1262 Novel Ionizable Lipids Derived from 2-Hydroxypropylamine and Spermine for mRNA-LNP Delivery

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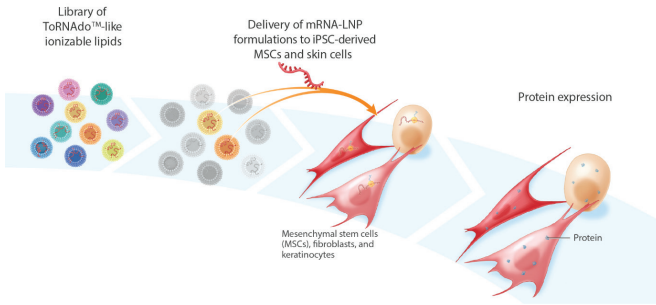
Lipid nanoparticles (LNPs) containing cationic or ionizable lipids offer several advantages compared to other vehicles for nucleic acid delivery and have seen expanded clinical use with the introduction the COVID-19 mRNA vaccines and in treatments for genetic diseases. However, poor targeting, insufficient cellular uptake, and low endosomal release currently limit the use of lipid nanoparticles as efficient delivery systems for next-generation gene therapies. To address these limitations, we designed, synthesized, and formulated novel ionizable lipids as LNPs to optimize mRNA delivery to cells. We developed a library of 12 ionizable lipids containing hexyl 2-hexyldecanoate lipid tails and hydrophilic headgroups derived from spermine or 2-hydroxypropylamine. Nanoparticle formulations incorporating these ionizable lipids were prepared at a molar ratio of 50:38.5:10:1.5 (ionizable lipid: cholesterol: DSPC/DOPE: DMG-PEG_{2000}) with mRNA encoding GFP at an N/P ratio of 3. For all the lipids tested, a mean diameter ranging from 100-150nm was measured by DLS. All of the lipids showed encapsulation efficiencies between 70-80%, comparable to LNPs formulated using FDA-approved lipids ALC0315 and DLin-MC3-DMA. LNPs were administered to iPSC-derived MSCs, primary human fibroblasts, and keratinocytes. Microplate imaging showed peak GFP fluorescence between 40- and 48-hours post-transfection. We identified novel ionizable lipids that produced LNPs exhibiting lower mean particle sizes, higher loading efficiencies, and enhanced GFP expression compared to LNPs incorporating ALC0315 or DLin-MC3-DMA. These results indicate that lipids containing elements of spermine and 2-hydroxypropylamine headgroups may serve as components of next-generation, lipid nanoparticle-based gene therapies and assist in the rational design of ionizable lipids for mRNA delivery.



1263 Correcting Multi-Exonic DMD Deletions Using a CRISPR/Cas9- Based Knock-In System

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Duchenne muscular dystrophy (DMD) is the most common pediatric muscular dystrophy, with an incidence rate of 1 in 5,000 males due to its recessive X-linked mode of inheritance. Multi-exonic deletions that disrupt the open reading frame of the gene and stop dystrophin synthesis are the most common cause of the condition. The absence of dystrophin causes damage to build up in muscle cells during periods of contraction and relaxation. Continuous damage leads to increased muscle wasting in cardiac and skeletal muscles, significantly reducing life expectancy. A curative therapy for DMD deletions is within reach due to advances in CRISPR/Cas genome editing tools. We propose a novel strategy using these systems to correct the *DMD* patient deletion of exons 52-54 through targeted DNA knock-in of the deleted exons, termed homology-mediated end-joining (HMEJ). The proposed system was initially tested *in-vitro* on mouse myotubes harbouring the *Dmd Δ52-54* deletion followed by transition to a mouse model harbouring the same mutation. In both models, we have observed significant restoration of the wildtype transcript and dystrophin-positive fibres. The results of this project will demonstrate the efficiency of this gene editing strategy and determine its therapeutic capacity.

1264 Cationic Diblock Polymeric Nanoparticles Encapsulate and Deliver a Full-Length 20 kb cDNA to Neuronal Cells

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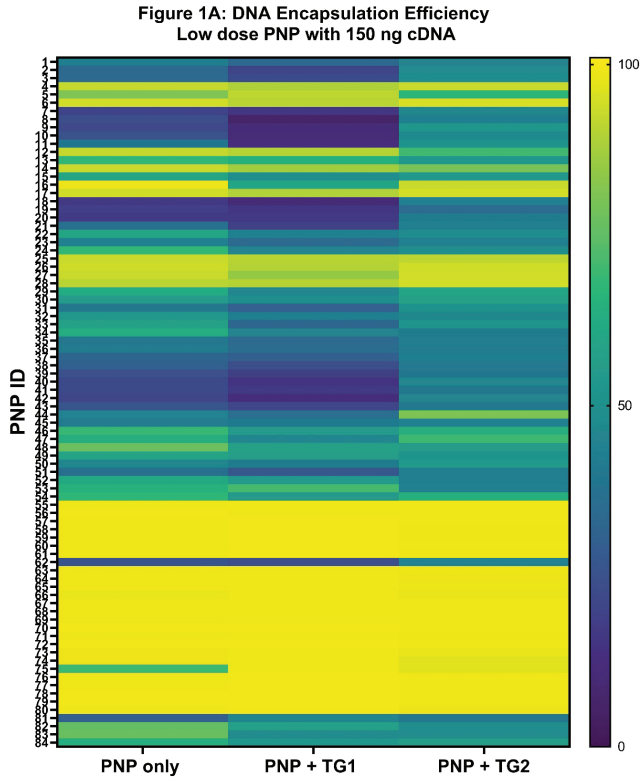
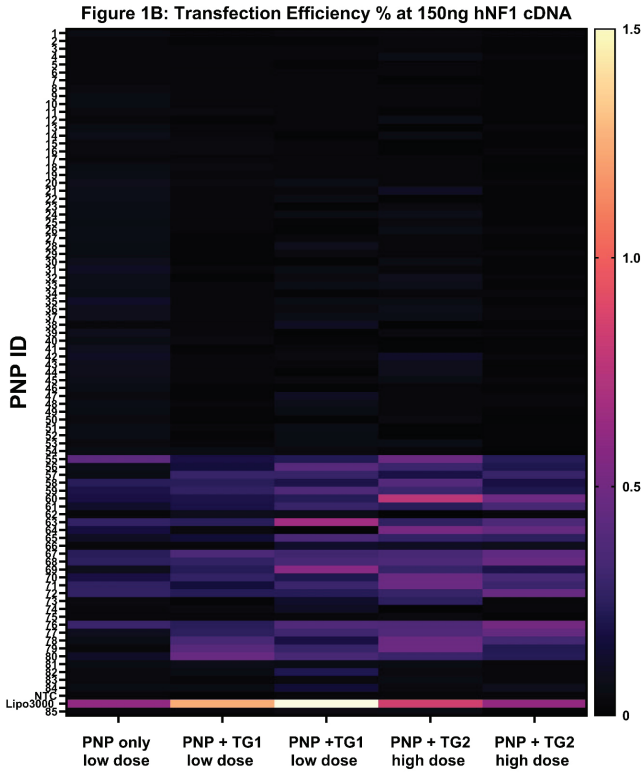
Reconstituting functional proteins via exogenous cDNA delivery to treat genetic diseases such as Neurofibromatosis 1 (NF-1) presents a promising treatment modality which, due to its wide applicability, has high potential for near-term clinical translation. This is currently blocked because of the lack of a suitable delivery vector that can encapsulate the large NF1 cDNA, which at 8.5 kbp is out of reach for viral vectors. In addition, there are no existing strategies that can achieve safe systemic targeted delivery to cells such as Schwann cells (SC) in the nervous system. To overcome these challenges, we used self-assembled cationic di-block polymeric nanoparticles (PNP) to encapsulate and deliver the NF1 cDNA plasmids to SCs *in vitro*. The key advantages of the PNPs proposed here are: large cargo capacity, access to extensive structural diversity, ability to be functionalized for targeting, ease of preparation, and stability during storage. A newly developed human NF1 cDNA plasmid (19553 bp) was used as cargo. This hNF1 plasmid contains a previously described NF1 cDNA with mini-intron under the CMV promote in the pCEP4 vector backbone and also encodes for a 2A-eGFP such that it expresses GFP. Further, a human telomerase reverse transcriptase (hTERT) immortalized primary human NF1 null SC line [CRISPRed out NF1 (ipn97.4 #24 NF1 -/-)] was used for all *in vitro* testing to ensure clinical relevance. Six different monomers with varying logP and pKa values were used to design a diverse combinatorial library of hundreds of PNPs with both active and passive targeting functionalities. A high-throughput synthesis and screening platform was used to test the PNPs *in vitro* in Schwann cells. cDNA loading efficiency was characterized using a RiboGreen dye exclusion assay. Transfection efficiency was quantified by analyzing cells with positive GFP expression on flow cytometry and cytotoxicity was quantified using a live-dead stain. Our results demonstrate that the cDNA encapsulation efficiency and transfection efficiency of the PNPs in the Schwann cells was modulated by the following factors: molecular weight, monomer type, monomer pKa, and the cDNA to PNP mass ratio. A subset of our PNP library, represented in the bottom 1/3 of Figure 1A and Figure 1B were best at encapsulating the cDNA and transfecting Schwann cells *in vitro*. Figure 1 shows heat maps of the cDNA encapsulation efficiency and transfection efficiency for the PNP library with passive and two active targeting groups (TG1 and TG2). The cell viability was over 90% for all tested PNPs. The results presented here provide a basis for future optimization of polymeric delivery vectors that can transfect this challenging cell target and point to an exciting opportunity for the field of non-viral gene delivery in general and neuronal cells in particular.





1265 Cardiac AAV Gene Therapy is Enabled after Repeat Injection by Temporary Coronary Occlusions in a Pig Model of Heart Failure

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Introduction: Cardiac gene therapy with adeno-associated virus (AAV) has been challenged by low efficacy in human trials. A significant barrier to intravascular delivery is the formation of neutralizing antibodies (NAbs), which may preclude repeat dosing. Using a pig model of heart failure, we previously showed that AAV transgene expression via intracoronary (IC) administration can be significantly enhanced by brief simultaneous coronary artery and coronary sinus occlusions (CAO + CSO delivery). We hypothesized that this technique could also overcome NAb inhibition. Methods: Yorkshire pigs (38.2±1.1 kg, n=9) pre-screened for low or negative NAbs against AAV6 were enrolled. Three groups (n=3/group) were designated to receive IC delivery of AAV6 encoding for luciferase (AAV6.Luc) one week after acute myocardial infarction (MI). One group received AAV6 (encoding for GFP, AAV6.GFP) 3-4 weeks prior to MI as a first injection in order to induce NAbs against AAV6. This group then received AAV6.Luc by CAO + CSO as a second AAV6 injection. The other groups (without prior AAV6 injection) received AAV6.Luc by CAO + CSO or by slow continuous delivery as reference. Luciferase assay was assessed for expression in heart regions one month later. NAb assay and ELISpot assay with detection of interferon (IFN)-γ were conducted to evaluate humoral and cellular immune responses pre- and post-AAV6.Luc injection. Results: In the delivery groups without previous AAV6 injection, CAO + CSO delivery resulted in significantly higher luciferase expression in all cardiac tissue regions (infarct, infarct-border, and remote posterior epi- and endocardial) compared to continuous delivery (>700-fold global difference). In the group that received previous AAV6.GFP injection, NAb assay reflected an increase in the NAb titer one month following AAV6.GFP (first) injection and prior to AAV6.Luc (second) injection. Even with the presence of induced NAbs, CAO + CSO delivery led to positive luciferase expression within infarct and infarct-border tissues (Figure). IFN-γ detection by ELISpot demonstrated increased number of spots after each AAV6 injection, with the highest detection one month after AAV6.Luc delivery, suggesting increased cellular immune response following second AAV6 injection. Conclusion: This study addresses the possibility of achieving AAV gene expression in the presence of neutralizing antibodies using a novel delivery technique, while also suggesting upregulation of cellular immunity after repeat dosing.

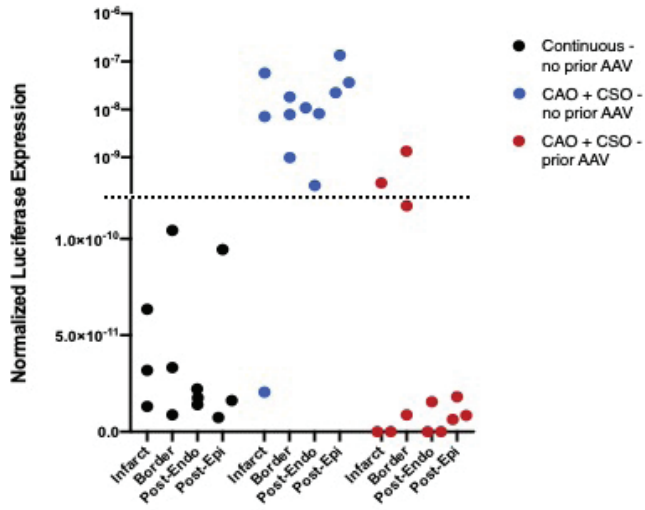

Figure. Luciferase expression in the heart by AAV immunity and delivery group. AAV6 encoding for luciferase was delivered by intracoronary injection one week post-MI to three groups (n=3/group): Continuous infusion without prior AAV6 injection (reference), delivery with coronary artery and coronary sinus occlusions and a cardiac support device (CAO + CSO) without prior AAV injection, and CAO + CSO with prior AAV injection (second dose). Negative values are represented as 0.0.

1266 Improved *In Vivo* Hemophilia A Gene Therapy Based on A Novel Immune-Escape Lentiviral Vector System

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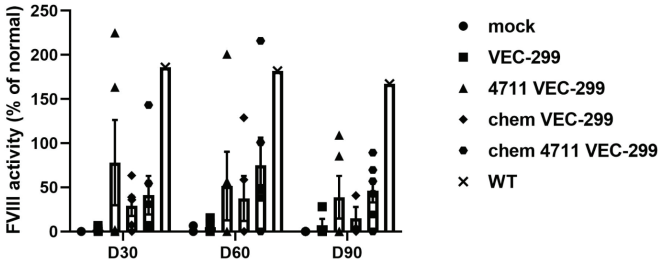
Hemophilia A (HA) is an X-linked monogenic coagulation disorder in the intrinsic coagulation cascade resulting from deficiency of the factor VIII (FVIII, F8) gene. Gene therapy is a promising treatment for HA. However, a major limitation to direct intravenous (iv) lentiviral vector (LV) injection has been the poor biodistribution and fast clearance of the LV particles from the circulation by professional phagocytes and/or innate immune activation. We have developed an advanced LV system for intravenous (iv) F8 gene therapy based on a novel B domain deleted F8-BDD gene, with fully restored N-glycosylation sites (F8-299) in the B domain and an endothelial-specific (VEC) promoter which demonstrate increased coagulation function with reduced inhibitory effect (Gong et al. J Biol Chem 2021, DOI: 10.1016/j.jbc.2021.101397). To reduce immune clearance of the iv injected LVs, we used a dual immune-shield strategy for LV packaging referred to as "4711-LV-VEC-299", which is based on increased CD47 expression on the packaging cell surface to suppress phagocytosis of the assembled virus particles, and the incorporation of cytomegalovirus US11 and ICP47 in the packaging cells to induce degradation of major histocompatibility complex class I (MHC-I). LV iv injection was performed after a non-myeloablative conditioning (chem) approach with busulfan (3.2 mg/kg), cyclophosphamide (100 mg/kg) and dexamethasone (5 mg/kg) or

a non-conditioning approach. Day 0 refers to the day of LV iv injection of transduction unit of $8x10^7$/mouse. The vector copy number (VCN) in the blood of chemo-conditioned "chem VEC-299" and "chem 4711-VEC-299" groups reached 7% and 20% on day 7, respectively. It gradually reduced from day 15 to 60 to below 1%. Functional assay based on chromogenic assay using F8 mouse plasma at day 30, 60, and 90 after treatment demonstrated high F8 activity in groups "4711-VEC-299" and "chem 4711-VEC-299", approaching the therapeutic range (~50% of the normal range) from day 30 to 90, whereas the "VEC-299" and "chem VEC-299" mice showed low F8 activities (see Figure). The 4711-VEC-299 LV treatments without chemo-conditioning also showed F8 activity in plasma, indicating the success of immune escape of the "4711" LV. Further *in vitro* and *in vivo* characterization of the 4711 LV is underway to illustrate the mechanism of immune evasion. These early results demonstrated that iv 4711-LV-VEC-299 without chemo-conditioning in the F8 knockout mice was feasible and exhibited high and long-lasting coagulation function and immune evasion. The novel 4711-LV system could be a potentially safe, convenient and effective HA gene therapy tool in clinical applications.



1267 Formulation and Process Development for Stable Lyophilized AAV8 Drug Products

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Recombinant adeno-associated virus (rAAV) vectors have emerged as the leading gene delivery platform for treatment of monogenic disorders due to their superior safety profile, diverse tissue tropisms, and relatively mild immunogenicity. Currently for clinical and commercial products, rAAV vectors are typically formulated and stored at ≤-65°C as frozen liquid solutions. However, long-term storage of frozen liquid AAV products are often far from ideal because it may result in shorter drug product shelf-life compared to recombinant protein-based biologics, and also present challenges for supply chain and inventory management. Consequently, there is great interest in developing robust lyophilized AAV drug products that are stable at refrigerated condition (2-8°C). In this study, we systematically evaluated the key formulation excipients required for stable lyophilized AAV products including buffers, salts, cryoprotectants, surfactants, and bulking agents. We optimized the concentrations as well as the ratios between the key excipients. This led to the identification of the lead formulation that demonstrated short-term in-solution stability at 25°C and upon lyophilized, sufficient long-term stability at 2-8°C with the elegant cake appearance of the lyophilized drug product. Contrary to what was reported recently, we showed that by using a robust lyophilization process based on optimization of critical process parameters, we successfully promoted crystallization of mannitol and achieved an elegant product cake with low moisture content. Furthermore, our study demonstrated that mannitol has no detrimental effect on product quality and stability. The systemic approach we demonstrated in this study could be applied to streamline the development of lyophilized AAV gene therapy products with various target transgenes and capsid serotypes.

1268 A Novel Three-Plasmid Packaging System for High-Yield and rcAAV-Free rAAV Production

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The HEK293-triple plasmid transfection platform is most widely used for rAAV clinical manufacturing. Despite countless advantages, the relatively low yield and generation of replication-competent AAV (rcAAV) particles impede its wider applications for future gene therapies. To overcome these bottlenecks, we designed a novel three-plasmid packaging system, Higher-Expression Recombinant AAV (HERA) system, which increased the rAAV yield by 5 to 10-fold and reduced rcAAV to an undetectable level. We implemented the following strategies to improve rAAV upstream yield: (1) screening and reconstitution of promoters for rep and cap gene; (2) selection and insertion of heterologous intron DNA sequences; (3) nucleic acid point-mutation to regulate pre-mRNA splicing; (4) optimization of rep/cap expression stoichiometry. To eliminate rcAAV, the following strategies were evaluated: (1) Homology reduction to prevent recombination events; (2) Stuffer insertion to prevent co-packaging; (3) recombination element separation; (4) transfection reagent compatibility screening. The HERA system has been evaluated in Neurophth's suspension HEK293 platform for multiple preclinical and clinical batches, across different serotypes and genes of interest. Herein, we present process and quality attributes summaries of historical batches to demonstrate the HERA system not only significantly reduced manufacturing scale but also achieved an improved quality profile. Utilizing the HERA system, the upstream rAAV yields were enhanced across various projects to the range of 3E14 vg/L to 1E15 vg/L, while the upstream rAAV yields were in the range of 3E13 vg/L to 2E14 vg/L when using the traditional plasmid packaging approach. Consequently, the HERA system has the potential to drastically reduce the demand for manufacturing scale and production cost due to the 5 to 10-fold improvement in rAAV yield. Meanwhile, the HERA system produced rcAAV-free drug products with comparable purity and impurities profiles to drug products produced using the classical plasmid packaging system.

1269 A Highly Sensitive NanoLuciferase Reporter Enables Efficient Functional and Genetic Biodistribution Assessment of AAV in Mice

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AAV based gene therapy is a rapidly growing field and has the potential to cure a wide variety of monogenic diseases by delivering replacement for genes harboring undesirable mutations. To maximize efficacy and safety of the treatment, the delivery of the replacement genes must target specific tissues. Unfortunately, naturally occurring AAV serotypes display broad tissue tropism. Engineering of AAV capsids has been a favorable approach to enhance specificity toward target tissues while minimizing off-target delivery. AAV variant selection often comprise of large sample sets and a complex workflow that requires automation. Each animal is injected with a variant AAV capsid library, followed by the isolation, purification and sequencing of DNA from each AAV infected tissue. Herein we report a simple, fast and automated workflow to enable extraction, purification and quantification of viral DNA from tissue samples in a high throughput fashion. To demonstrate our workflow, we generated AAV9 viruses expressing a NanoLuc® reporter (AA9-NanoLuc) to examine tissue tropism in animals via bioluminescent live imaging. The NanoLuc® reporter displays superior brightness enabling detection at day 7 post injection. Additionally, it is small enough to be packaged into both single stranded and self-complementary AAV systems. In our study, we divided 40 mice into 4 groups of 10 (5 males and 5 females) and injected varying amounts of AAV9-NanoLuc into each group (Negative - 0 GC/Kg, Low - 1e10 GC/Kg, Medium - 1e11 GC/Kg, High - 1e12 GC/Kg). Two weeks after injection, bright and dose dependent bioluminescent images were captured via live whole-body imaging. Next, tissues were collected, and total DNA was extracted and purified using the Maxwell nucleic acid extraction system. Total DNA quantity and purity were assessed, followed by determination of AAV viral titers by normalization to house-keeping gene *TERT* using digital droplet PCR. We found AAV titers of several organs correlated with their luminescent signals. The viral titers accumulated in targeted tissues were also strongly dependent on the injection dose. In summary, we report a versatile NanoLuc® reporter that is bright and well suited to examine tissue tropism in live animals. In addition, we provide a simple, single day workflow which enables high throughput compatible viral titer determination of AAV infected tissues. The combination of our reporter and our proposed workflow would advance efficiency and effectiveness when screening AAV capsid libraries.

1270 The Optimization of AAV9 Dosage and Fluorescein Concentration in Direct Vagus Nerve Injection to Treat Autonomic Dysfunction

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We previously reported that a direct vagus nerve injection of AAV9/GAN (scAAV9/JeT-GAN) in combination with lumbar intrathecal (IT) delivery added benefit to the treatment of autonomic dysfunction caused by giant axonal neuropathy (GAN) (ASGCT-2020, Abstract#936). In that study, a single dose of 2.6E12 vg/rat IT simultaneously combined with a dose of 6.5E11 vg/rat directly into the vagus nerve was conducted in GAN-KI (knock-in) adult rats. The AAV9/GAN vector injected into the vagus nerve was mixed with luxol fast blue (LFB) dye to enable visualization and monitor the injection. In the present study, our goal was to identify the optimal dosage to transduce critical regions of the autonomic nervous system by injecting AAV9/GFP (scAAV9/CBh-GFP) into the vagal nerve of wild type adult Fischer 344 rats. To achieve this goal, we mixed five different doses (6.5E11, 2.5E11, 7.6E10, 2.5E10, or 2.5E9 vg/rat) of AAV9/GFP with 1% LFB, dosed each rat with a single unilateral injection of AAV9/GFP into the left vagus nerve at cervical region, and harvested autonomic gangaila along the vagus nerve and medulla for immunohistochemistry analyses 28 days post administration. We observed a dose dependent transduction variation in nodose ganglia of vagus nerve, dorsal motor nucleus, solitary tract and nucleus, and nucleus ambiguous of caudal medulla. The two highest doses (6.5E11 and 2.5E11 vg/rat) resulted in abundant and equivalent transduction efficiency in the cervical vagus nerve, nodose ganglia and brainstem sites of caudal medulla, leading us to conclude 2.5E11 vg/rat as the minimal dosage required to achieve maximal transduction efficiency of these critical regions of autonomic nervous system. Considering that LFB cannot be used as a tracking dye in humans to our knowledge, we further evaluated a range of fluorescein concentrations for their impact on transduction and feasibility to enable tracking of the injection fidelity in real time. Using a constant dose of 2.5E11 vg/rat AAV9/GFP, mixed with four different fluorescein concentrations (0.001, 0.002, 0.004, and 0.008) prior to vagal nerve injection into wild type Fischer 344 rats, we identified 0.004% fluorescein as the most optimal concentration to monitor injection fidelity. Taken together, these studies have identified practical parameters for optimal injection of AAV9 directly into the vagus nerve to target critical regions of the autonomic nervous system, which are prerequisite to facilitate the translation of this approach to larger animals and humans.

1271 Combining CAR T Cell Therapy with Novel STING Agonist for Successful Treatment of Solid Tumors

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While chimeric antigen receptor T cells (CART) have achieved therapeutic success in hematological malignancies, similar results have not been observed in patients with solid tumors to date. Hurdles in CART therapy targeting solid tumors include insufficient cell trafficking into the tumor, as well as the immunosuppressive tumor microenvironment (TME) which hampers engineered T cell function. A possible strategy to increase efficacy and therapeutic response of CART in solid tumors could be the combined use with IMSA101, a novel stimulator of interferon genes (STING) agonist, to effectively modify the tumor microenvironment prior to engineered T cell administration. To test this approach, we established two syngeneic flank tumor models using immunocompetent C57BL/6 mice. Mice were either implanted with PDA7940b, an immunologically cold murine pancreatic ductal adenocarcinoma (PDA) cell line derived from KPC ($Kras^{LSL.G12D/+}p53^{R172H/+}$) mice, or with B16, an immunogenic murine melanoma cell line. Approximately one week after tumor cell injection, animals were treated with intratumoral IMSA101 alone, intravenous CART alone, IMSA101+CART, or were left untreated for control. At the time point of peak CART expansion, the TME was analyzed by flow cytometry of tumor single cell suspension in addition to pathological assessment and immunohistochemistry (IHC) of bulk tumor tissue. RNA was extracted from intratumoral CART for gene expression and pathway enrichment analyses, and mouse serum was analyzed for cytokines. In addition, a xenograft flank tumor model was established using NOD/scid/IL2r$\gamma^{-/-}$ (NSG) mice and human PDA AsPC-1 cells to characterize the effects of IMSA101 on human CART. Tumor clearance was seen in all B16 tumor-bearing mice, as well as in some PDA7940b tumor-bearing mice when both treatments were combined. This resulted in significantly improved overall survival when compared to mice treated with CART alone or IMSA101 alone in both syngeneic models. Treatment-related weight loss or clinical signs of distress were not observed in any of the mice. More CART were detected in tumors of IMSA101+CART-treated mice when compared to mice treated with CART alone. Importantly, gene expression analysis of intratumoral CART between these two cohorts showed activation as well as effector T cell signatures in CART+IMSA101-treated mice. In addition, more pro-inflammatory cytokines were detected in serum of mice receiving combination treatment in both syngeneic models. IMSA101 also enhanced human CART anti-tumor efficacy, resulting in significantly improved overall survival of NSG mice. Further analyses in xenograft tumor models are currently ongoing. In summary, intratumoral administration of IMSA101 improved CART trafficking into the tumor, induced intratumoral CART activation, and significantly enhanced overall anti-tumor efficacy and overall survival in two syngeneic flank tumor models and in an immunodeficient model with human CART, suggesting direct and indirect mechanisms of enhanced antitumor immunity. These promising results paved the way for development of the combination approach into the clinic.

1272 Comparison of CAR-T Cell-Mediated Cytotoxicity Assays with Suspension Tumor Cells Using High-Throughput Plate-Based Image Cytometry Method

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In the recent decade, chimeric antigen receptor (CAR)-T cell therapy has revolutionized strategies for cancer treatments due to its effective clinical efficacy and response for B cell malignancies. Currently, there are six CAR-T cell products available in the market including Kymriah®, Yescarta®, Tecartus®, Breyanzi®, Abecma®, and Carvykt™. The success of CAR-T cell therapy has stimulated the increase in the research and development of various CAR constructs to target different tumor types. Therefore, a robust and efficient in vitro potency assay may be beneficial to quickly identify potential CAR gene design from a library of construct candidates. Traditionally, in vitro CAR-T cell-mediated cytotoxicity is assessed using release assays such as 51Cr (radioactivity), calcein (fluorescence), and LDH (enzymatic). Image cytometry methodologies have been utilized for various CAR-T cell-mediated cytotoxicity assays using different fluorescent labeling methods, mainly due to their ease-of-use, ability to capture cell images for verification, and higher throughput performance. In this work, we employed the Celigo high-throughput plate-based Image Cytometer to evaluate and compare two CAR-T cell-mediated cytotoxicity assays using GFP-expressing or fluorescent dye-labeled myeloma and plasmacytoma cells. In regards to the performing time and E:T ratio-dependent CAR-T cell-mediated cytotoxicity assays, the GFP-based method demonstrated higher sensitivity in detecting the level of cytotoxicity when compared to the CMFDA/DAPI viability method. We have established the criteria and considerations for the selection of cytotoxicity assays that are fit-for-purpose and to provide meaningful results for the specific testing conditions. * All trademarks, service marks, trade names, and brand names are the property of their respective owners.

1273 Addressing Key Challenges to Enable Global Access to Gene Therapies

Markus J. Haindl

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Gene Therapies are currently where Monoclonal Antibodies were 25 years ago and we need a shift of technical development and analytical characterization paradigms as well as transformative innovation for reliable supply, more sustainable cost of manufacturing as well as enhanced molecular understanding of these next generation therapeutics. This presentation provides insights to our approaches

and scientific advancements to transform manufacturing and improve molecular understanding by analytical characterization in order to enable global access to AAV based therapeutics.

1274 Optimization of Transfection Reagent Formulation and Experimental Conditions to Maximize the Percentage of Full Adeno-Associated Virus Capsids

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Adeno-associated virus (AAV) has emerged as a leading platform for gene delivery for the treatment of a variety of human diseases. Generating high titer AAV at large-scale is technically challenging; however, process optimization (improved vectors, cells, media, culture conditions and transfection reagents) has resulted in robust improvements in AAV yield. Increasing the total yield of AAV manufactured is critical to moving gene therapies into mainstream medicine. Historically, viral genomes measured by qPCR or dPCR was paramount; however, the importance of the quality of the AAV, including the percentage of capsids containing AAV genomes as measured by the percentage if full capsids, is now considered a critical quality attribute (CQA) in generating therapeutic AAV. In response to this need, Mirus Bio is developing a new transfection formulation that provides multi-fold increases AAV genome titers over existing PEI based reagents while also increasing the percentage of full capsids. During these studies, using AAV8 as a model system, we have found that the transfection reagent used to generate recombinant AAV preparations in suspension 293 cells has a strong effect on not only the genome titer, but also on the virus quality as measured by the percentage of full capsids. Other factors affecting the percentage of full capsids were assessed with top candidates. These included: cell density, complex formation time, DNA dose (µg pDNA per ml of culture), and transfection reagent-to-DNA ratio. When using a high performing transfection formulation, alterations in transfection and/or cell growth conditions can have a larger effect on the percentage of full capsids compared to genome titer. The data presented in this poster illustrates how changes in the upstream transfection process can affect the AAV8 titer, and/or the quality of the virus produced which can positively affect downstream processing.

1275 Large-Scale Production of High-Quality Recombinant Adeno-Associated Virus Using Short-Term Zonal Ultracentrifugation

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Background: Purification of recombinant adeno-associated virus (rAAV) is required for the safe and efficient transduction in rAAV-based in vivo gene therapies, because rAAV products contain full-genome (functional), intermediate, and empty rAAV particles as well as host-cell proteins. However, the current standard method for rAAV purification at the laboratory scale (up to 180mL) is based on two-day ultracentrifugation with a cesium chloride (CsCl) density-gradient protocol, limiting large-scale purification of functional rAAV for clinical usage. In addition, the infectivity decreases when the rAAV is incubated in CsCl over time. Therefore, in this study, we sought to develop a short-term purification method for large-scale rAAV production at high purity by using a zonal ultracentrifuge. Methods: rAAV9 encoding ZsGreen1 was collected from culture supernatant (without cell lysis) 5 days after plasmid transfection. Large-volume rAAV-containing culture media (up to 1,000mL) and escalating densities (2 or 4 steps) of CsCl solutions were separately placed in the zonal rotor, respectively. Following large-scale density-gradient ultracentrifugation of rAAV, the samples were fractionated automatically. These samples were evaluated by rAAV genome copies in quantitative PCR, rAAV capsid protein amounts in western blotting, and ZsGreen1 transduction efficiency in flow cytometry. The purity of rAAV was analyzed by analytical ultracentrifuge (AUC) and transmission electron microscope (TEM) with phosphotungstic acid stain. The genomic DNA regions packaged in rAAV particles were detected by droplet digital PCR (ddPCR) using various primers and probes targeting the whole vector genome. Results: We performed 2-step CsCl density-gradient ultracentrifugation with a zonal rotor, allowing for a large volume (900-1000mL) of rAAV purification as well as shorter ultracentrifugation for 4-5 hours with a steeper density-gradient in the area of rAAV fractions, as compared to our control method of 4-step density-gradient (300mL) for 10 hours. rAAV capsid proteins were detected in 2 of the fractions, one of which also contained the peaks of rAAV capsid, genome copies, and ZsGreen1 transduction, demonstrating a separation between functional full-genome and non-functional empty rAAV particles. The intermediate particles would be included between these fractions. We detected a single AUC peak with separate sedimentation coefficients for fractions of full-genome (about 90S) and empty (about 60S) vectors, demonstrating a high-purity separation of rAAV. Interestingly, the whole genome regions were detected at similar levels in full-genome rAAV by ddPCR; however, ITR signals were 2.5-2.9 fold higher than the ZsGreen1 signal in the empty particles, suggesting that ITR fragments can be packaged in 'empty' particles. In addition, we purified large-volume rAAV after removing small molecules and increasing concentration by simple pretreatment (up to 1.13x1014 v.g.). Conclusion: We developed a 2-step density-gradient ultracentrifugation method with a zonal rotor in 4-5 hours, allowing for large-scale purification of functional full-genome rAAV. We also improved the fractionation system for short-term purification. Our large-scale rAAV purification method would be applicable for considerable in vivo experimentation and clinical investigation.

1276 Uncovering Nucleic Acid Payload and Size of Lipid Nanoparticles via A High-Throughput Single Nanoparticle Analyzer

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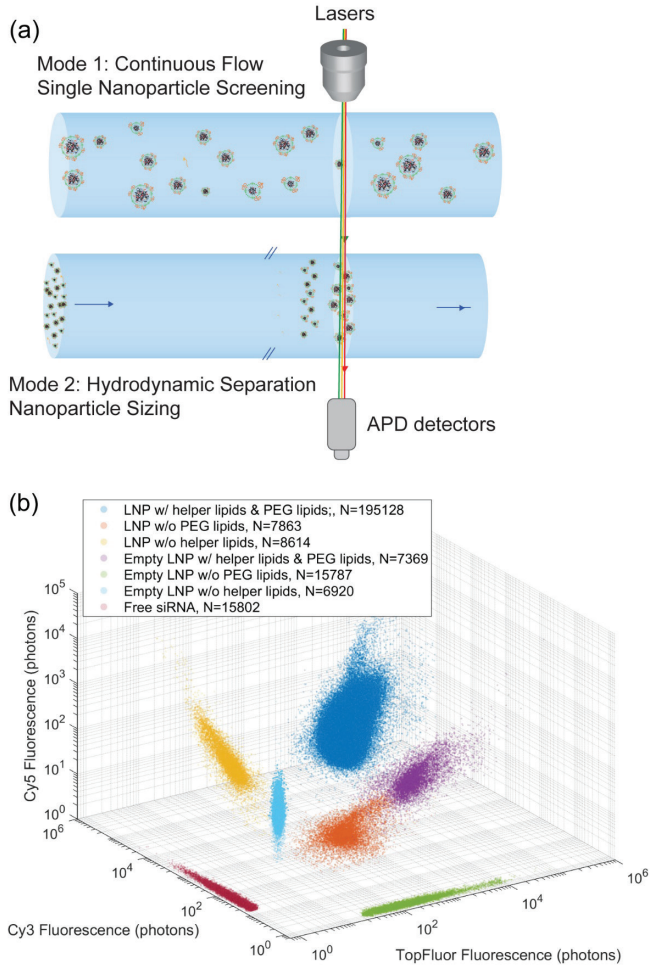
Introduction: Lipid nanoparticles (LNPs) have broad applications as vehicles for RNA vaccines and therapeutics. However, the fundamental understanding of the structure-property-function relationships of the LNP system falls behind technological advancement and could hamper further development in more critical settings. In particular, there is limited understanding of the formation of subpopulations during manufacturing, single-nanoparticle nucleic acid and lipid payload level, or the correlation between nanoparticle payload and size. Here, we assessed the siRNA payload and content loading and distribution of two lipid components in a benchmark siRNA LNP formulation and the size of LNPs at the single-nanoparticle level. These results could provide more insights into the LNP assembly mechanism. **Methods**: We used Cylindrical Illumination Confocal Spectroscopy (CICS), a technique recently applied for LNP characterization[1] to examine the LNPs with fluorescent-labeled Cy3-siRNA, TopFluor-PC lipids, and Cy5-PEG lipids (Fig. 1a). The LNPs were injected continuously or in sample plug for Single Molecule Free Solution Hydrodynamic Separation (SML-FSHS)[2] in a microfluidic channel (Fig. 1a). A commonly referenced benchmark formulation was studied with the dosage of ionizable lipid DLin-MC3-DMA, cholesterol, the helper lipid DSPC, and DMG-PEG2000 at a molar ratio of 50:38.5:10:1.5. The LNPs were formulated by rapid mixing in a T-junction at pH 4.0 (acetate buffer) and dialyzed against PBS at pH 7.4. **Results**: The 3-color labeled siRNA LNPs were screened at the single nanoparticle level by CICS, and their signals were analyzed based on the fluorescence coincidence criteria. The fluorescence of all the identified events (>250,000 particles) was plotted in 3D space (Fig. 1b). 80.7% of the LNP signals were siRNA LNPs with both helper lipids and PEG lipids; 3.3% were siRNA LNPs without PEG lipids; 3.6% were siRNA LNPs without PC helper lipids; 3.0% were empty LNPs with both helper lipid and PEG lipid; 6.5% were empty LNPs without PEG lipids; 2.9% were empty LNPs without PC helper lipids. There was a total of 12.4% empty LNPs. CICS reported an average of 29.0 siRNAs per LNP for this formulation. Next, SML-FSHS was applied to analyze the hydrodynamic size of the siRNA LNPs at three PEG lipid concentrations (4.5%, 1.5%, 0.5% PEG). SML-FSHS yielded larger particle sizes (59 nm, 87 nm, and 115 nm at peak) compared to CryoTEM (28 nm, 41 nm, and 60 nm at peak) for LNPs at 4.5%, 1.5%, 0.5% PEG, respectively, as SML-FSHS measures the hydrodynamic size including the corona of the LNPs, whereas CryoTEM reveals the size of LNP cores.



Conclusions: This CICS-based LNP composition analysis technique allows for quantitative assessment of siRNA LNP subpopulations such as empty LNP percentage, lipid distribution, and siRNA payload and distributions. It also measures the hydrodynamic size of LNPs, incorporating the hydrodynamic separation. Overall, the method offers a powerful single nanoparticle analysis tool to study the structure-property-function relationships of LNPs. **Reference**: [1] *Nat. Commun.* **2022**, *13* (1), 5561. [2] *J. Am. Chem. Soc.* **1016**, *138* (1), 319-327.

1277 Development of Rationally Designed CAG-Based Promoters for Use in Gene Therapy

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Background: The promoter is an essential cis-regulatory element in any DNA-based gene therapy. It directly controls gene transcription and thereby therapeutic protein expression. In the context of genetic medicines, stronger promoter activity may enable a lower vector dosage of the given gene to achieve therapeutic effect, reducing safety risks associated with high vector dosages, as well as reducing

manufacturing costs. To date, an overwhelming majority of promoters used in gene therapy clinical trials are based on cellular CAG or viral CMV promoters. We endeavored to engineer new CAG-based promoters with the aim to increase potency and reduce size to create an improved set of strong, durable constitutive promoters amenable to gene therapy applications. **Methods:** The CAG promoter is a synthetic hybrid promoter consisting of the CMV immediate early enhancer and the promoter, the first exon, and a modified first intron of the chicken beta actin gene. We rationally designed a series of 82 new CAG promoter variants by systematically introducing modifications to each of the promoter elements and tested them in different *in vitro* and *in vivo* models. **Results:** In our library of CAG promoter variants, 51 are smaller than the original CAG, of which 22 are fewer than 1000 base pairs in length. In HEK293T cells, 67 CAG promoter variants were found to be stronger than the original CAG with the strongest promoter exhibiting 13-fold improvement in potency. Two CAG promoter variants, based on improved *in vitro* activity and smaller size (~40% size reduction), were administered by tail vein injection into C57BL/6 mice. Expression in the liver improved by up to 4-fold compared to the original CAG promoter. **Conclusions:** Through our promoter engineering platform, we successfully created a library of promoters based on CAG, a proven effective and safe promoter, with increased potency at driving gene expression and that displays promise for the next generation of gene therapy development.

1278 Utility of Cryofluorescence Tomography (CFT) as an Unbiased Method to Describe AAV Biodistribution

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The use of adeno-associated viruses (AAV) as gene delivery vectors has vast potential for the treatment of many severe human diseases. A small group of intensively studied AAV capsids have been propelled into pre-clinical and clinical use, and more recently, marketed products; however, many less-studied, naturally occurring capsids may also have desirable properties (e.g. potency differences, tissue tropism, reduced immunogenicity, etc.) that have yet to be thoroughly described. Within this under-studied group of capsids is AAV7, a potentially therapeutic capsid for which tropism studies are limited, and direct, head-to-head comparisons with well-described capsids have not been performed. We sought to characterize the biodistribution of this capsid more fully by several methods including quantitative PCR, immunofluorescence (IF) and immunohistochemistry (IHC), and whole-body imaging via cryofluorescence tomography (CFT) as an unbiased method to look for novel regions of transduction. CFT of whole mice was performed three weeks after IV administration of AAV7 or AAV9 carrying an EGFP transgene driven by CAG, or co-administration of AAV7-EGFP and AAV9-tdTomato. This was compared to traditional biodistribution analysis of vector genome copies and expressed RNA transcripts. Superiority of AAV9 in mouse skeletal muscle was shown over AAV7 and was confirmed via CFT and IF. Cardiac transduction by AAV7 trended higher than AAV9 in all studies. AAV7 and AAV9 both transduce the mouse brain with equal efficiency according to PCR-based biodistribution data; however, by CFT and IHC, cell type differences were found within the brain, with AAV7 being localized mostly around blood vessels within the brain, and AAV9 widely transducing astrocytes and neurons. A surprising finding from CFT was the presence of fluorescence in facial structures, some of it highly concentrated, for both AAV7 and AAV9. As this is not typically an area sampled by traditional biodistribution methods, this finding has been largely overlooked. In particular, the mandible showed the highest intensity of EGFP fluorescence of any other tissue for both AAV7 and AAV9. The incisor also had measurable EGFP fluorescence. As the mouse mandible is surrounded by muscle tissue that likely was transduced, we conducted an IHC study to further ascertain detailed information about the transduction seen in and around mouse facial structures and investigate tropism differences between AAV7 and AAV9. Surprisingly, strong transgene expression was observed throughout the head, as verified by IHC, including regions of the cranial sinuses, teeth, and mandible for both AAV7 and AAV9, an attribute that has not been previously evaluated via traditional biodistribution methods. These data will help build a broader structure-function knowledge base in the field, present capsid engineering opportunities, and enable the use of novel capsids with unique properties. Overall, CFT represents an unbiased method to evaluate novel AAV capsids, eliminating initial bias in tissue sampling and giving precedent for further investigation of tissues of interest.

1279 Evaluating Pharmacology and Efficacy of Delandistrogene Moxeparvovec in Young and Aged DMD^{MDX} Rats

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Gene transfer therapy is a promising treatment for patients with Duchenne muscular dystrophy (DMD). Delandistrogene moxeparvovec (previously known as SRP-9001) is an investigational recombinant adeno-associated viral vector-based gene transfer therapy designed to compensate for missing dystrophin in DMD by delivering the SRP-9001 dystrophin transgene, which encodes a shortened, engineered dystrophin protein that retains key functional domains of the wild-type protein. The purpose of this preclinical study was to evaluate the myocardial efficacy and safety of intended commercial process delandistrogene moxeparvovec material in DMD^{MDX} rats. DMD^{MDX} rats are a valuable alternative DMD animal model, as they demonstrate cardiac dysfunction that recapitulates cardiac dysfunction of patients with DMD. We performed systemic, intravenous delivery of intended commercial process delandistrogene moxeparvovec material in young (21-35 days old) and aged (3-5 months old) DMD^{MDX} rats. The older rats demonstrate a more severe phenotype in terms of fibrosis or cardiac disease progression. Rats received a dose of $1.33x10^{14}$ vg/kg. Ambulation activity was recorded via the Photobeam Activity System Open Field. Echocardiograms and histological analysis of fibrosis were used to evaluate cardiac disease.

Data from 12 weeks and 24 weeks post-systemic delivery demonstrated no evidence of cardiac toxicity. Importantly, there were no deaths attributed to treatment. Compared with the saline control, intended commercial process delandistrogene moxeparvovec material increased ambulation and vertical activity in young DMD^{MDX} rats and improved cardiac function. Protein expression was broadly distributed across skeletal muscle, the diaphragm, and the heart. Taken together, these findings confirm expression in cardiac muscle, as expected, and support the myocardial efficacy and safety of delandistrogene moxeparvovec. Further evaluation of cardiac disease phenotypes at 12 and 24 weeks post-systemic delivery utilizing several indicators of cardiac function will also be presented. This study was funded by Sarepta Therapeutics.

1280 P2Y12 is a Novel Druggable Target for Blocking Macrophage Myofibroblast Transition Driven Cancer Associated Fibroblast Formation in Lung Carcinoma

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Macrophage-Myofibroblast Transition (MMT) is a newly discovered Smad3-dependent mechanism for the direct generation of protumoral cancer-associated fibroblasts (CAF) from tumor-associated macrophages (TAM). Better understanding of its regulatory mechanism would uncover novel therapeutic targets for developing precision medicine against CAF-driven non-small-cell lung carcinoma (NSCLC). By conducting high-resolution single-cell RNA-sequencing with bone marrow-derived macrophages (BMDM), we identified P2Y12 as a novel Smad3 direct target gene in the cells undergoing TGF-β-driven MMT *in vitro*. We found that macrophage P2Y12 level significantly correlated to the CAF abundance and associated with poorer overall survival of NSCLC patients. Genetic silencing of macrophage P2Y12 markedly suppressed MMT-driven myofibroblast formation and protumoral effectors *in vitro* and *in vivo*. Encouragingly, pharmaceutical inhibition of P2Y12 with FDA-approved P2Y12 inhibitor Clopidogrel effectively blocked MMT-driven CAF formation and cancer progression in mice bearing syngeneic lung carcinoma LLC as well as human NSCLC xenograft *in vivo*. Thus, we successfully identified macrophage P2Y12 as a druggable target for precisely targeting MMT-driven protumoral CAF formation in lung carcinoma.

1281 Improved Transgene Expression from GenY AAV Serotype Vectors in Human Cells *In Vitro* and in Murine Hepatocytes *In Vivo*

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We have previously reported that the distal 10-nts in the AAV2 D-sequence share partial homology with the glucocorticoid response element (GRE) ½ binding site, and that the AAV2 genomes in which the distal 10-nts with were replaced with the 15-nts consensus full-length GRE site mediated up to ~6-fold increased transgene expression in primary human skeletal muscle cells when packaged into AAVrh74 vectors (*Mol. Ther.*, 30: 237-238, 2022). In the current study, we examined whether similar increased efficacy of transgene expression could also be achieved with other AAV serotype vectors. To this end, HeLa cells were transduced with WT and GenY AAV2 vectors, and transgene expression was evaluated 72 hrs post-transduction. As can be seen in **Figure 1A**, The extent of transgene expression was ~2-fold higher with GenY AAV2 vectors, it was further increase by ~2-fold following dexamethasone-treatment, suggesting that the observed increase in transgene expression is indeed due to activation of the GR pathway, and that the GRE ½ site is potent enough to increase the transduction efficiency in response to dexamethasone-treatment. Transduction efficiencies of WT and GenY AAV2 vectors were also evaluated in C57BL6/J mice following intravenous delivery *in vivo*. These results, shown in **Figure 1B**, also document ~3-fold increase in transgene expression from GenY AAV2 vectors. Interestingly, sequence alignment of D-sequences of AAV1 through AAV6 revealed the absence of the GRE ½ site in the rest of the AAV serotypes (**Figure 1C**). The 15-nts consensus full-length GRE site was inserted in the AAV6 D-sequence to generate GenY AAV6 vectors. As can be seen in **Figure 1D**, GenY AAV6 vectors also mediated up to ~3-fold increase in transgene expression in HeLa cells compared with the WT AAV6 vectors, and it further increased by ~3-fold following dexamethasone-treatment. Since glucocor-ticoids, such as predni-solone, are routinely used prophylactically to mitigate the AAV vector-mediated host immune responses in clinical trials, our data suggest that insertion of the full-length GRE site in the recombinant AAV genomes may be a simple strategy to achieve increased trans-gene expression at lower doses of AAV vectors.

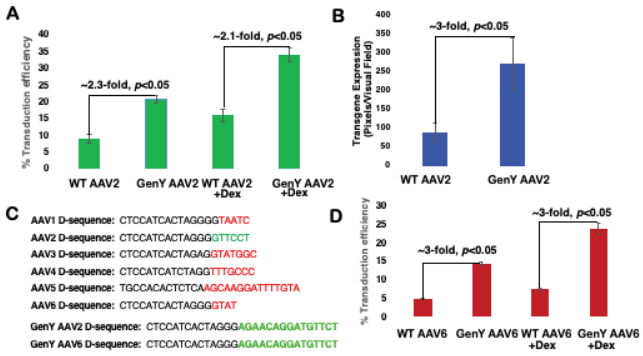

Figure 1: A. Transduction efficiency of WT and GenY AAV2 vectors in HeLa cells. Cells were transduced in triplicates at 500 vgs/cell of each vector, with or without pre-treatment of cells with 10 μM dexamethasone, and transgene expression was quantitated by flow cytometry. **B.** WT and GenY AAV2 vectors were injected intravenously at 1x10^{11} vgs/mouse (n=6 each) and transgene expression in liver sections was visualized under a fluorescence microscope 4-weeks post-injections. **C.** Sequence alignment of D-sequences in AAV1 through AAV6 serotypes. The GRE ½ site (green font) is present only in AAV2 D-sequence. 15-nts consensus full-length GRE site (bold green font) was inserted in AAV2 and AAV6 D-sequences to generate GenY AAV2 and GenY AAV6 vectors. **D.** Transduction efficiency of WT and GenY AAV6 vectors in HeLa cells. Cells were transduced in triplicates at 3,000 vgs/cell of each vector, with or without pre-treatment of cells with dexamethasone, and transgene expression was quantitated as described above.

1282 CRISPR Base Editing Approach to Treat Duchenne Muscular Dystrophy by Skipping DMD Exon 45

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Duchenne Muscular Dystrophy is an X-linked degenerative muscular disease that first manifests as muscle weakness and that progresses rapidly leading to paralysis and, ultimately, death of the patients in their 3rd decade of life. At the molecular level, Duchenne is caused by mutations in dystrophin, a protein that is critical for maintaining the stability of the muscle membrane by linking the cytoskeleton of the muscle cells with the extracellular matrix. While there is no cure for Duchenne, exon skipping with antisense oligonucleotides (AONs) has emerged as a highly promising therapeutic approach that interferes with the spliceosome to exclude exons containing mutations from mature mRNA, which creates a truncated dystrophin that ameliorates the symptoms of Duchenne. Currently, most methods of exon skipping require direct delivery of exon skipping oligonucleotides to the muscle cells, an approach that has several limitations, including that it is inherently transient and that it requires recurrent invasive and costly procedures over the lifetime of the patient. We have recently developed CRISPR-SKIP, a technology that utilizes base editors for disrupting the conserved AG dinucleotide within the splice acceptor of target exons. This method overcomes the problems associated with conventional exon skipping by achieving efficient and permanent exon skipping in the target cells. In our current work, we demonstrate that CRISPR-SKIP can effectively disrupt the splice acceptor of exon 45 in the dystrophin gene, a strategy that can be utilized to restore the dystrophin reading frame in patients with deletions in exons 44 or 46, which comprise approximately 9% of cases of Duchenne. First, we tested a panel of base editors that we have created and we demonstrated that they can introduce targeted base modifications in up to 90% of the targeted alleles, which is anticipated to be sufficient to provide a functional correction of DMD. Additional experiments demonstrated that targeted genome editing led to efficient skipping in mRNA and restoration of dystrophin protein expression. Furthermore, the majority of the targeting guides utilized did not induce detectable off-target mutations. Finally, to enable in vivo correction of Duchenne, we developed a split intein base editing system that is compatible with AAV delivery. We demonstrate that split intein base editors fully recapitulate the activity of their full-length counterparts at the splice acceptor of dystrophin exon 45. More importantly, split base editors delivered by AAV in vivo following intramuscular or systemic injections efficiently edited the splice acceptor of DMD exon 45. In summary, this work describes a novel exon skipping strategy that relies on base editors to enable efficient skipping of exon 45 in the dystrophin gene as well as a platform for in vivo delivery of base editing tools for correcting Duchenne muscular dystrophy.

1283 Microfluidic Capillary Electrophoresis for Measurement of Full and Empty AAV Capsids

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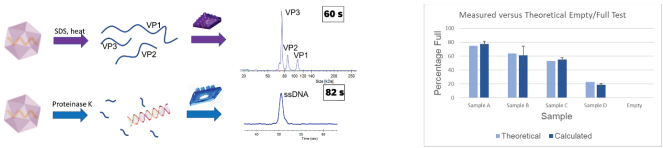
Adeno-associated virus (AAV) particles are intensively being investigated for therapeutic use in gene therapy due to low immunogenicity and ability for long-term gene expression in vivo. During development, testing strategies for the viral protein stoichiometry, capsid product purity, and ssDNA loading efficacy are used to monitor and assess AAV efficacy. The loading efficacy is observed as the percentage of capsids containing ssDNA (full capsids). While TEM and AUC measurements display high accuracy for empty/full measurements, these methods have high cost and time requirements. As such, there is a need for an accurate and high-throughput platform characterization system for empty/full measurement that requires low AAV volume input. Here we describe a microfluidic capillary electrophoresis method that measures AAV8 capsid proteins and ssDNA of samples alongside measurement of an AAV8 standard. Capsid protein and ssDNA area measurements are mathematically analyzed to afford the percentage full of AAV sample. The microfluidic system with AAV standard referencing method enables microliter sized samples to be analyzed in ~1 minute per sample and is concentration independent. Measurement and calculation of empty:full percentage of AAV8 samples with concordance to the expected values (within 12% of target) was achieved for sample mixtures of full and empty reference AAV particles. **Assay Overview and Experimental Setup** To determine the empty/full ratio, we began with a 10 µL fraction of AAV sample and 10 µL fraction of the AAV reference standard (75% full, UV-Vis). 5 µL of the standard and sample are mixed with a proteinase K/urea solution that upon heat treatment, digests the viral protein capsid. Microfluidic Capillary Electrophoresis (LabChip® GXII Touch™) is run using a single strand nucleic acid-binding dye for laser-induced fluorescence detection for ssDNA measurement. The remaining 5 µL of the AAV sample and reference sample are then mixed with 7 µL of a protein denaturing solution. Heat treatment and subsequent adjustment of the ionic strength prepare the solution for measurement of VP3 concentration. The percentage of full capsids was determined using an algorithm with inputs of the sample ratio of VP3 area, sample ratio of ssDNA area and the known percentage full content of the AAV8 reference standard. **Results**The average percentage full of duplicate measurements for Vigene AAV samples were found to be within 4.4% of the theoretical value for samples containing 50-75% full capsids, and all values obtained were within 12% of the theoretical value. The error for Vigene C sample was 18.8% (Target 23% full, measured 18.7% full). Sirion Biotech AAV8 samples were also made via mixing to produce different levels of full capsids. Analysis of these samples yielded an average error of 8.3% from expected. Control samples composed of 100% empty capsids did not show any measurable ssDNA signal above baseline and were calculated to be 0% full. A plot of calculated empty/full ratio versus the theoretical empty full ratio yields a linear regression R2 value of 0.96. **Conclusion** The microfluidic capillary electrophoresis-based method enables the rapid determination of the empty:full ratio for AAV8 particles. AAV8 samples can be assessed for empty/full ratio with protein and ssDNA measurements in ~1 minute per run. 10 µL samples with at least 1E12 VP/mL are recommended for use, however the exact

concentration of starting material does not need to be known. The LabChip® GXII Touch™ method provides a fast and cost-effective way to determine the empty/full ratio for AAV8 capsids.



1284 Optimizing Intravitreal Delivery to the Non-Human Primate Retina with Machine-Guided AAV Capsid Design

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To enable transduction of deeper retinal cell layers, current AAV-mediated ocular gene therapies require high doses to achieve clinically relevant efficacy which increases the risk of adverse inflammatory responses, or employ subretinal injections that must be performed by trained surgeons. More efficient cell-type targeted pan-retinal transduction via intravitreal (IVT) administration would allow for more effective therapies at lower and safer doses, broadening the reach of ocular gene therapies. Using machine-guided design we generated variant capsids with up to 80 times more efficient transduction of the neural retina in non-human primates (NHP) following IVT delivery as compared to AAV2. We then evaluated cell-type specific transduction patterns in the neural retina by single-nucleus RNA-seq. Relative to an AAV2-derived literature variant reported to have improved retinal transduction, these capsids demonstrated 2-6x improvement in retinal cell types including rods, cones, microglia and retinal ganglion cells. Extrapolating to a conservative dose of 8.0e10 vg/eye, we infer that these variants are capable of transducing a high proportion of cells across retinal cell types, for example up to 17% in retinal ganglion cells. The true proportion of transduced cells is likely even higher due to incomplete capture of transduction events in single nucleus sequencing. We selected one top capsid for further validation and injected it intravitreally into NHP eyes. Qualitative and quantitative in-life and histological readouts for transduction properties were performed based on reporter transgene expression. Histological readouts confirmed the variant's significantly improved transduction efficiency compared to AAV2-derived literature variants. These results show the utility of single-molecule based NGS-based readouts in both library and validation contexts and demonstrate the power of applying machine learning to capsid design.

1285 Gene Editing Therapy in a Humanized Mouse Model of Amyotrophic Lateral Sclerosis

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Background: Amyotrophic lateral sclerosis (ALS) is a late-onset fatal neurodegenerative disease with an incidence rate of approximately 2-5 per 100,000 people in Europe and the United States. The neuropathological hallmark of ALS is the degeneration of cortical and spinal motor neurons with common symptoms such as muscle weakness, twitching, cramping, dyspnea, and dysphagia, leading to respiratory failure, paralysis, and death, typically within 2 to 5 years of symptom onset. About 10% of ALS patients are familial, and most of them are caused by the mutations in genes such as *C9orf72, SOD1, TDP-43,* and *FUS*. The dysfunctional protein aggregated in neurons is central to the pathology of ALS, and 97% of ALS patients eventually show the accumulation of mutated TDP-43 protein in the cytoplasm. TDP-43 is a DNA/RNA-binding protein essential for normal functions of the upper and lower motor neurons. It was reported that *ATXN2* promotes the formation of stress granules involving TDP-43 protein, and knockdown of *ATXN2* effectively inhibits the formation of stress granule aggregates by TDP-43. Hence, *ATXN2*-targeting gene editing is one of the potential treatment strategies for ALS. **Methods**: To explore the therapeutic effect of single AAV vector packaging high-fidelity Cas12i (hfCas12Max) and gRNAs targeting *Atxn2* (gAtxn2), we administrated AAV-hfCas12Max-gAtxn2 in postnatal day 0-1 humanized *TDP-43* transgenic mice via intracerebroventricular (ICV) injection (Fig. 1A). The indel efficiency was tested by PCR plus NGS sequencing. ATXN2 protein level in the cortex was evaluated by western blot and immunofluorescence staining. The lifespan of humanized *TDP-43* transgenic mice with or without AAV-hfCas12Max-gAtxn2 was also observed daily. **Results**: In the neonatal humanized *TDP-43* transgenic mice, the indel ratio of *Atxn2* gene reached 46.1% (Fig. 1B), and the knockdown efficacy of ATXN2 protein level was up to 86.8% in the cortex at 2 weeks post infection with AAV-hfCas12Max-gAtxn2, compared with that in control mice (Fig. 1C-E). Median survival of humanized *TDP-43* transgenic mice injected with high-dose AAV-hfCas12Max-gAtxn2 (29.75±0.5 days) was 5 days longer compared with humanized *TDP-43* transgenic mice injected with PBS (25.29±0.99 days), representing a 20% extension of survival (Fig. 1F). **Conclusions:** Our findings provide a strong foundation and promising approach for the clinical application of AAV-hfCas12Max-gAtxn2 to treat ALS, which may potentially increase the lifespan of patients with ALS.

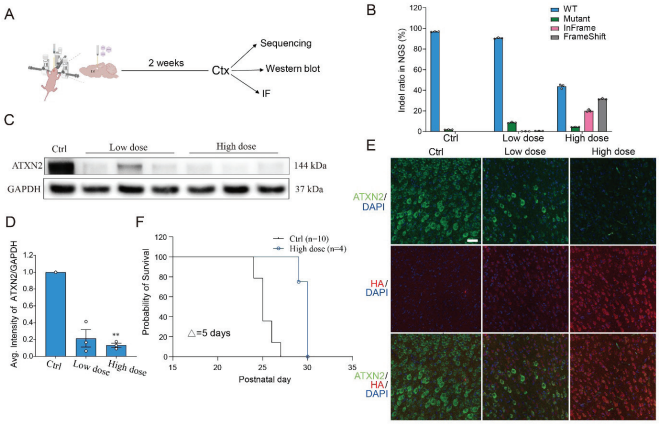

Figure 1. The treatment effect of AAV-hfCas12Max-gAtxn2 in the humanized *TDP-43* transgenic mice

1286 Combined +58 and +55 *BCL11A* Enhancer Editing Yields Exceptional Efficiency, Specificity and HbF Induction in Human Hematopoietic Stem Cells

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Targeting the *BCL11A* erythroid enhancer by gene editing is a promising approach to fetal hemoglobin (HbF) induction for β-hemoglobinopathies. HbF levels vary widely among individuals. We hypothesize that maximizing HbF induction potential could maximize clinical benefits. Here we compared CRISPR-Cas9 endonuclease editing of the *BCL11A* +58 enhancer with alternative gene modification approaches, including +55 enhancer editing alone or in combination with the +58 enhancer, as well as editing targeting the *HBG1/2* promoter -115 BCL11A binding site and transduction by an shRNA knocking down the *BCL11A* transcript in erythroid precursors. We found that combined editing of the +58 and +55 enhancers with 3xNLS-SpCas9 and two sgRNAs resulted in the most potent HbF induction (52.4%±6.3%) of the tested approaches (*BCL11A* +58 editing alone, 29.1%±3.9%; *BCL11A* +55 editing alone, 34.8±5.1%; *HBG1/2* promoter editing, 34.1% ±5.4%; shmiR-*BCL11A*, 32.2%±4.4%; mock, 7.6%±3.4%). Based on bulk and clonal analyses, we found that disruption of core half E-box/GATA motifs at both the +58 and +55 enhancers was associated with greatest enhancer chromatin inaccessibility and HbF induction, whether by small indels, interstitial 3.1 kb deletion or 3.1 kb inversion. Combined editing of +58 and +55 enhancers was compatible with HSC self-renewal and multilineage repopulation in primary and secondary xenotransplantation. We developed conditions using a MaxCyte electroporation instrument achieving mean 97.3±1.8% gene edits in 3 engineering runs at clinical scale, with similar results at small-scale with plerixafor-mobilized HSPCs from sickle cell disease (SCD) donors or G-CSF mobilized PBMCs from transfusion-dependent beta-thalassemia (TDT) donors, including 94.2%±4.4%, 99.5%±0.3% and 91.8%±6.3% of gene edits in engrafting cells from NBSGW 16 week mouse bone marrow of healthy, SCD and TDT donors respectively. Off-target analyses by pooled amplicon sequencing of 1288 candidate off-target sites for the +58 and +55 targeting sgRNAs, nominated by a range of computational (CRISPRme) and experimental (GUIDE-seq and ONE-seq) methods, did not identify reference genome off-target edits at a sensitivity of 0.1% allele frequency. We found the fraction of micronuclei was increased in cells electroporated with RNP following 24 or 48 hours of cytokine stimulation but not in unstimulated cells. We observed ~9-fold enrichment of chr2p in micronuclei after 24 or 48 hours of pre-stimulation cytokine culture prior to gene editing, but did not observe enrichment of chr2p in micronuclei in cells edited without pre-stimulation culture, suggesting that chromosome mis-segregation could be avoided by editing quiescent HSCs. We found editing cells without pre-stimulation culture was associated with undetectable long deletions and rearrangements as compared to editing after 24 or 48 hours of pre-stimulation culture. We observed similar HbF levels, editing and engraftment potency without as compared to with cytokine culture across mobilization type (G-CSF or plerixafor), donor disease status (healthy and SCD), and sgRNA target sequence. Together these results suggest that combined *BCL11A* +58 and +55 enhancer editing without cytokine culture produces highly efficient on-target allelic disruption, heightened HbF induction capacity compared to alternative approaches, preserved long-term multilineage engraftment potential, and absence of evident genotoxicity, under clinically relevant SpCas9 RNP electroporation conditions.

1287 Characterization of Genomic Heterogeneity in rAAV Preparations Using Short- and Long-Read Next Generation Sequencing

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Three approved gene therapies within the last 6 years demonstrate that recombinant adeno-associated viral (rAAV) vector products are an accepted modality for delivering therapeutic transgenes to correct monogenic disorders. As commercial scale is needed to support the demand for AAV therapies there may be a need to manufacture large, reproducible batches of AAV drug products that are safe, pure, and potent. Biological outcomes associated with these attributes may be influenced by the genetic heterogeneity within populations of rAAV vector preparations. Molecular profiling of the genetic heterogeneity within rAAV vector preparations can provide insight into batch-to-batch reproducibility and inform on vector modifications that can improve these attributes. We applied long-read and short-read next generation sequencing (NGS) as orthogonal methods for molecular profiling of rAAV vector preparations. Our results demonstrate that

genetic composition can be influenced by choice of AAV capsid, method of producing AAV vectors, vector design, and downstream purification method. Learnings from rAAV molecular profiles will be processed by a unique artificial intelligence (AI) platform which can inform iterative vector designs.

1288 Development of AAV Transduction and Potency Assays to Evaluate Quality Attributes of ASC618, a Second Generation AAV8-Based Hemophilia A Gene Therapy

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ASC618 is a second generation of AAV8-based gene therapy for Hemophilia A under clinical development. It utilizes an optimized liver-specific promoter to express an engineered human Factor VIII variant (ET3-FVIII) that is secreted with high efficiency from hepatocytes. To support the ongoing Phase 1/2 clinical trial that evaluates the safety, tolerability, and preliminary efficacy of ASC618 (NCT04676048), we have established robust analytical methods to assess the quality attributes of ASC618 GMP lots. We have developed a ddPCR-based AAV transduction assay to evaluate relative infectivity of ASC618 GMP lots in HepG2 cells (a human liver cancer cell line), compared to a reference standard (ASC618 Engineering Lot). During method development of the transduction assay, we observed that the transduction efficiency, measured by a ddPCR assay specific for theET3-FVIII transgene in ASC618-transduced HepG2 cells, strongly correlated with the level of ET3-FVIII protein in the cell culture media. Therefore, a single preparation of ASC618-transduced HepG2 cell culture can be used for both the transduction assay (using HepG2 cell pellets) and the potency assay (using cell culture supernatant), which streamlines the analytical workflow and reduces the amount of drug substance (DS) and drug product (DP) required for testing. The potency of ASC618 GMP lots relative to that of a reference standard (ASC618 Engineering Lot), which is evaluated by two assays that we have developed: a Potency-ELISA assay and a Potency-Activity assay, which XYNTHA® Antihemophilic Factor VIII (Recombinant) was used to generate standard curves for both assays. In pre-qualification experiments, the ASC618 Engineering lot was applied as Reference Standard and used to prepare QC samples with target ratio of potency (TRP) of 0.5 (50% QC), 1 (100% QC), 1.5 (150% QC) and 2 (200% QC) respectively. Both the Potency-ELISA Assay and the Potency-Activity Assay exhibited accuracy within 30% for TRP 0.5 - 2. In addition, by dividing the Potency-Activity result (IU/mL) by the Potency-ELISA result (IU/mL), we observed that the ET3-FVIII in the HepG2 cell culture supernatant has relative activity of 87.8% ± 20.4% to XYNTHA® Antihemophilic Factor VIII (Recombinant). By using the transduction, Potency-ELISA, and Potency-Activity assays, three independent batches of ASC618 drug substance (DS) or drug product (DP) have been tested. The relative transduction confirmed high consistency with the relative potency in both ELISA and Activity assays.

1289 Promoter Triage for Gene Expression & Function Optimization

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Optimizing lentiviral vector designs to increase titer, decrease production costs, and increase potency is an active area of exploration especially for vectors entering large scale manufacture. Here, we tested the effect of different promoters on multiple CAR and transgenic TCR constructs for their effect on titer, integration, expression, and in vitro function. To achieve this, we developed a scaled down mid-throughput lentiviral vector production platform to produce multiple constructs in tandem with sufficient titers for functional screening. We found that the MND promoter was able to ubiquitously produce high titers of lentiviral vector and generated a high level of expression across all CAR and TCR constructs tested. Interestingly, EF1αL consistently produced lower titers compared to MND based constructs, but when transduced at the same MOI produced high levels of expression in T cells. Conversely, the SFFV and MSCV promoters showed varying titers and expression dependent on the encoded gene of interest. Comparing viral copy number and expression data, we were able to evaluate transduction efficiency and surface expression levels separately for each gene of interest. Unsurprisingly, cytotoxicity and function of each construct tested was highly correlated to the expression level on the cell surface in most cases. These data will help guide future construct design and help streamline clinical candidates towards scale and up feasibility studies.

1290 Optimization of a Virus-Free CRISPR-Cas9 Based Platform to Produce Allogeneic Chimeric Antigen Receptor (CAR) T Cells

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Autologous T cells expressing the chimeric antigen receptor (CAR) represented a breakthrough in hematological malignancies' treatment, leading to remission rates as high as 90%. Despite the impressive success, the custom-made pattern of such therapy restrains its widespread applicability due to defective cellular characteristics of intensively pretreated patients, complex production logistics and cost. Allogeneic T cells from healthy donors became an excellent alternative as a starting material for CAR T cell manufacture, enabling a staggered production of ready-to-use doses. Challenges of the resulting HLA-mismatched CAR T cell therapy can be addressed through T cell receptor (TCR) knock-out using genome editing technologies to prevent the graft-versus-host disease development. Here we described an optimization process of the CRISPR/Cas9 tool for a streamlined virus-free CAR T cell manufacture. With so, we targeted a transgene integration into the *TRAC* (*T Cell Receptor Alpha Constant*) locus, consequently disrupting the TCR. For the CRISPR/Cas9-mediated knock-in, we provided a homology-directed repair template (HDRT)

containing a second-generation anti-CD19 CAR or a mCherry sequence flanked by homology arms to the desired cut site in the human *TRAC* locus. This sequence was PCR amplified and purified, resulting in a highly concentrated double-stranded DNA HDRT, which was electroporated with the Cas9 ribonucleoproteins (RNPs) into healthy donors' T cells. Cells were assayed on days 5 and 11 post-electroporation for CAR expression and killing efficiency, respectively. We observed a 1.3-fold (±0,2) improvement in mCherry knock-in by adding the negatively charged polymer poly-L-glutamic acid (40 µg) that binds to RNPs and reduces aggregates (**Fig. 1A**). Additionally, by culturing the electroporated cells with increasing concentrations of an NHEJ inhibitor, we achieved a 1.3 (±0,1) to 2-fold (±0,02) increase in the editing efficiency (**Fig. 1B**), suggesting a favorable condition for the HDR. We then electroporated T cells with the CAR HDRT (3.2 kb length, 25 µg) using the established optimized conditions. We got high levels of TCR knock-out (91,5±0,4%) and CAR knock-in (83,7±4,0%) five days after electroporation (**Fig. 1C**), with the viability reaching ~60% on day 7. The on-target CAR gene integration was confirmed through an "in-out" PCR assay on the genomic DNA using primers specific to the *TRAC* locus and CAR transmembrane domain. In vitro potency of CAR T cells was measured by coculturing them with GFP-expressing RS4-11 cells ($CD19^+$). We observed a consistent control of the tumor cell growth at a 1:1 effector-to-target ratio (**Fig. 1D**), demonstrating the robust antitumor function of the CAR T cell products. Combined, the data provide a proof-of-concept for the virus-free manufacturing process of allogeneic anti-CD19 CAR T cells, enabling CAR knock-in and TCR knock-out in a single-step approach.

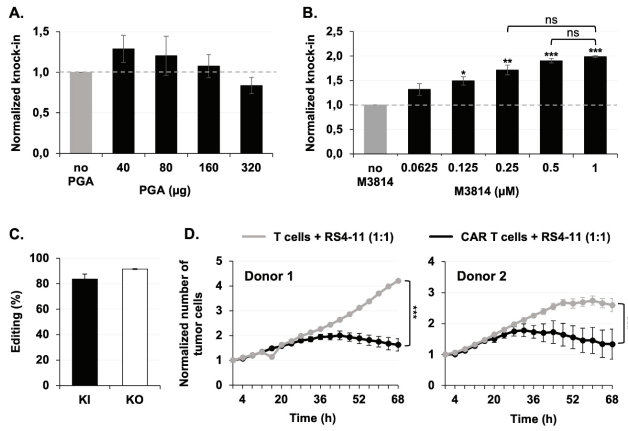

Figure 1. Improvement in mCherry knock-in rates using the anionic polymer poly-L-glutamic acid (PGA, **A**) and an NHEJ inhibitor (M3814, **B**). (**C**) Editing rates (CAR knock-in, KI; TCR knock-out, KO) 5 days after electroporation. (**D**) Tumor cell growth inhibition was observed for a 1:1 effector-to-target ratio. ns≥0,05; * p≤0,05; ** p≤0,01; *** p≤0,001.

1291 Development of Metabolically Reprogrammed HER2-CAR T Cells Suitable for Treating Breast Cancer

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HER2-targeted therapy is an established method for treating HER2-positive breast cancer. HER2 antibody-drug conjugate (ADC) is the newest additions to HER2-targeted therapy with Trastuzumab emtansine and Trastuzumab deruxtecan being recently approved by the FDA. Trastuzumab deruxtecan has shown to be effective in treating HER2-low breast cancer, allowing it to be the first targeted therapy for HER2-low breast cancer approved by the FDA. Although HER2 ADC drugs show effectiveness in killing HER2-positive breast cancer, the vast majority of HER2 ADC drugs result in a variety of side effects, with most being high grade or serious side effects. HER2 ADC's safety profile is similar to chemotherapy in several adverse events (AE), with two AEs of special interest (left ventricular dysfunction and interstitial lung diseases/pneumonitis) occurring more frequently in HER2 ADC drugs than it does in chemotherapy. Among these AEs, lung toxicity is an important safety concern of HER2 ADC drugs. Therefore, there is a need for a novel effective HER2-targeted therapy with lower toxicity. Remarkably, recent clinical studies demonstrated that the second generation HER2-CAR T cell with CD28 co-stimulatory domain showed lower toxicity with little incidents of grade 3 or higher adverse side effects, while having certain antitumor activity in early phase of clinical studies of HER2-positive Sarcoma and Glioblastoma. In this study, we developed and demonstrated the effectiveness of a metabolically reprogrammed HER2-CAR (MR.HER2-CAR) T cells that overexpress a secreted adenosine deaminase 1 (ADA1) fused with an anchor and CD26 (the receptor of ADA1) on the cell surface and. The ADA1 converts adenosine to inosine, allowing for the MR.CAR T cells to overcome adenosine-mediated immunosuppression and to provide inosine for CAR T cell growth. ADA1 is a cytoplasmic protein and conditionally secreted under stress, suggesting that ADA1 activates CAR T cells as a trans-signaling in a tumor specific manner. The overexpressed CD26 induced a rich chemokine receptor profile, allowing the CAR T cells to traffic to solid tumors. We further demonstrated that these MR.HER2-CAR T cells are more effective in killing HER2-low breast tumor cells than unmodified HER2-CAR T cells. Thus, MR.HER2-CAR T cell therapy is a promising candidate for a novel HER2-targeted therapy that is effective while having low toxicity.

1292 AAV Mediated Expression of a Chimeric Ion Channel in Combination with Its Ligand Varenicline Demonstrates a Dose Response Improvement in Pain Hypersensitivity in a Rat Model of Trigeminal Pain

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Chemogenetics is a method that provides selective control of cellular function by installing an engineered receptor that renders a targeted cell population sensitive to a small molecule ligand. KT-74863 is an AAV vector designed to drive expression of an engineered ligand-gated ion channel that is activated by the FDA approved drug varenicline to open a chloride-conducting ion channel that results in decreased neuronal excitability. KT-74863 in combination with varenicline (KT-74863-VT) is being developed for the treatment of trigeminal neuralgia. Trigeminal neuralgia (TN), also known as tic douloureux, is a rare, chronic and debilitating facial pain disorder that can be difficult to treat. Approved medications are poorly tolerated and lose their effectiveness over time, while surgical options are often complex, can be highly invasive and result in high rates of sensory loss. The pain is thought to be due to damage of the trigeminal roots or ganglion that results in hyperexcitability of the trigeminal nerve, giving rise to pain paroxysms. Control of hyperexcitability of neurons is affected primarily by the activity of membrane associated ion channels, whereby influx of anions through these channels leads to neuronal hyperpolarization and a decrease in pain. KT-74863-VT is expected to decrease trigeminal hyperexcitability following focal administration to the trigeminal ganglion and is being developed as a one-time treatment option for patients with TN who are refractory to medication. The preclinical efficacy and durability of KT-74863-VT was evaluated in the rat infraorbital nerve chronic constriction injury (IoN-CCI) model, an established model of trigeminal pain. Two treatment paradigms were tested: IoN-CCI surgery before or after KT-74863 administration. Efficacy was measured using a series of von Frey hairs applied to the whisker pads to determine the escape threshold (ET). Transgene expression was also measured in the trigeminal ganglion. KT-74863-VT in IoN-CCI male and female rats demonstrated a dose responsive improvement in pain hypersensitivity and durability regardless of treatment paradigm. Efficacy was observed throughout the 6-month duration of the study. The escape threshold (ET) significantly improved in both male and female IoN-CCI rats with no significant differences in ET between sexes. In contrast, neither KT-74863 alone nor varenicline alone demonstrated reductions in pain sensitivity. Analysis of relative levels of KT-74863 viral DNA and GlyR mRNA demonstrated dose dependent DNA/mRNA expression in the injected trigeminal ganglion. Varenicline levels in all animals receiving treatment were within predictable levels at the time of sample collection.

1293 Through the Ups and Downs - Leveraging siRNA-Mediated Gene Knockdown to Influence the Productive Capacity of HEK293 Cells in rAAV Production

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Recombinant adeno-associated viruses (rAAV) have become one of the dominant forms of viral vectors for *in vivo* gene therapy. A growing body of work has focused on identifying new principles and methods to increase the yield in rAAV production. Despite this, many aspects of the molecular mechanisms underlying rAAV production by the host cell are still elusive. The complex networks of signaling pathways, messenger compounds and effectors are not yet fully understood and therefore not trivial to manipulate. Here we show how next-generation sequencing can be used to identify targets for cell line engineering of rAAV producer cell lines. Transcriptomic analysis was performed on RNA-Seq data from two different commercially available HEK293 cell lines. Cells were transfected with a two-plasmid system, collected 18 h afterwards and total RNA was sequenced. Bioinformatic tools such as DESeq2, Clusterprofiler and Reactome PA were employed for gene expression and pathway analysis to identify target genes that might affect the cellular production capacity for rAAV. In total, 4,008 genes were differentially expressed between the two cell lines. These genes belonged to 872 significantly enriched GO terms, covering numerous cellular mechanisms that were deemed relevant for rAAV production. One promising gene from this set was selected for assay establishment and as a proof-of-concept candidate. As a main aspect, we present the Design of Experiments-assisted steps towards siRNA transfection optimization for suspension cells, comparing different reagents, ratios, and conditions. The suspension-based assay is performed in a scale-down approach in 24-well plates. Based on the optimized protocol, we showed the effect of the siRNA-mediated gene knockdown on growth performance, transfection efficiency and rAAV titer. This work demonstrates how data-driven approaches can facilitate further developments in cell line engineering and help increase rAAV production capacities.

1294 High Throughput Viral Vector Characterization Using Density Gradient Equilibrium Analytical Ultracentrifugation (DGE-AUC)

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Introduction to viral vector characterization and quality control: Quality control of gene therapy vector like adeno-associated virus (AAV) relies on a battery of biophysical techniques such as Anion Exchange Chromatography (AEX), Transmission Electron Microscopy (TEM), UV spectroscopy, light scattering, mass photometry (MP) and Sedimentation velocity Analytical Ultracentrifugation

(SV-AUC); the last of which is considered the gold standard for quantification of empty vs partially loaded vs fully loaded AAV capsids (E/P/F ratio). Here, we present a new method called Density Gradient Equilibrium Analytical Ultracentrifugation or DGE-AUC to characterize Adenovirus, AAV and other gene therapy products.
DGE-AUC: DGE-AUC operates on the same principles as density gradient ultracentrifugation (DGUC): i.e, analyte particles are subjected to ultracentrifugation in the presence of a density gradient-forming material such as CsCl. At equilibrium, the CsCl forms a radial gradient of increasing density. Every analyte particle migrates to the radial position where the local density of CsCl corresponds to its own density. Thus, at equilibrium - empty, partially loaded and fully loaded capsids which have increasing density migrate to and accumulate in the form of bands at increasing radial positions.
Advantages of DGE-AUC and comparison to SV-AUC:
• ***Separation basis:*** DGE-AUC separates analytes only on the basis of particle density, while SV-AUC separates particles on the basis of sedimentation coefficient, which is a function of particle size, shape and density. Thus, DGE-AUC is an orthogonal technique to SV-AUC.
• ***Simplified analysis:*** The analysis of DGE-AUC data does not require specialized software (unlike SV-AUC). The output from a DGE-AUC experiment can be visually interpreted intuitively and analyzed similarly to a chromatogram. Quantification of different capsid species (E/P/F ratio) is achieved by identifying and integrating the peaks corresponding to the different species.
• ***Low sample consumption:*** DGE-AUC provides high resolution separation with significantly less sample (> 30-fold sensitivity) than SV-AUC.
• ***High throughput:*** DGE-AUC experiments can be completed within a couple of hours. Useage of 6 sector AUC centerpieces (as opposed to the 2 sector centerpieces used in SV-AUC) increases throughout. Here, we demonstrate a high throughput adenovirus packaging analysis experiment, running as many as 21 samples in 80 minutes.
• ***Flexibility with samples:*** SV-AUC requires relatively pure samples. DGE-AUC works with in-process dirty samples as well.
• ***No size limitation:*** SV-AUC is not suitable for large analytes such as Adenovirus and other large viral vectors that sediment rapidly without providing enough scans for analysis. DGE-AUC effectively has no size limitations w.r.t. particles and can be applied to these samples.
• ***Multiwavelength (MWL) experiments:*** SV-AUC experiments with MWL scans require more time for data acquisition, which is not always available for large samples that sediment quickly. MWL-DGE-AUC can acquire additional scans at different wavelengths after equilibrium has been established, which provide more fine-grained information on the capsid nucleic acid content.

1295 Determining the Efficacy and Mechanism of NKG2D-Based CAR T Cells

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Chimeric antigen receptor (CAR) T-cells are genetically modified to specifically target tumour-associated cell surface targets. Despite clinical success in patients with haematological cancers, their competence against solid tumours is hindered by an immunosuppressive environment, and further understanding of the functionality of these cells is required to improve potency and scope of treatable cancers. We have investigated the use of NKG2D as a device to target CAR T-cells against stress ligands that are commonly expressed on solid tumour cells. This concept was originally developed by Sentman et al. More recently, several other groups have explored the use of second-generation CARs targeted by NKG2D with a view to improve anti-tumour activity. Clinical development of NKG2D-targeted CARs is currently being led by Celyad Oncology in a number of indications. However, a systematic comparison between the function and mechanistic properties of NKG2D-targeted CARs has not been undertaken. In my project, I compared a panel of second generation NKG2D-based CARs, with differing structures and sources of signalling moieties. They were assessed for their ability to re-target T-cell specificity against solid tumour cell lines. *In vitro*, they were assessed for their ability to continuously re-stimulate on cell lines, exerting cytotoxicity and secreting pro-inflammatory cytokines for several cycles. Additionally, T cells were phenotypically characterised both in culture and after tumour cell stimulation. Anti-tumour activity was additionally assessed in preclinical models using NSG mice bearing established xenografts, using both intraperitoneal/intraperitoneal (i.p/i.p) and subcutaneous/intravenous (s.c/i.v) models. Tumour burden was assessed using bioluminescence imaging to monitor disease status for the i.p/i.p model, and using calliper measurements to define tumour growth in the s.c/i.v model. NKG2D-based CAR T cells were also subject to bulk RNA sequencing post stimulation, with the aim of uncovering mechanistic insights into the functionality of these CAR T cells, including mechanisms governing their differentiation, cytolytic capacity, intracellular signalling, and metabolic status. We will present a comparative analysis of NKG2D targeted CAR T-cells in which CAR configuration and the source of stimulatory domains have been modified to assess their importance in overall function.

1296 High Systemic Dosing of an Engineered rAAV9 with TLR9 DNA-Based Immunomodulatory Sequence Allows Higher and Persisting Transgene Expression in Rats

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Recombinant adeno-associated virus-derived vectors (rAAV) have become an essential part of the gene therapy landscape. Despite the growing successes, the immune response directed to the vector and/or the transgene product nevertheless appeared in patients as a major limitation for a successful clinical translation of systemic rAAV products. One way to overcome rAAV-directed immune response is to prevent their initiation through the inhibition of the innate immune system activation. Several pattern recognition receptors (PRR) have already been described to detect pathogen-associated molecular patterns (PAMPS) present either in the capsid or the viral vector genome itself. Among them, the Toll-like-receptor 9 (TLR9) is shown to play an important role

in the detection of unmethylated CpG dinucleotides contained in the vector genome. Moreover, its inhibition by an antagonistic sequence added to the rAAV viral genome is reported to have an immunomodulatory effect on transgene expression after gene transfer. Here, we evaluated the effect of a novel engineered rAAV9 genome carrying a TLR9 DNA-based immunomodulatory sequence (TLR9 DIMS) after high systemic vector dosing. After *in vitro* validation, our TLR9 DIMS sequence was inserted in the vector genome of a rAAV9 cassette expressing the reporter gene Green Fluorescent Protein (GFP) and intravenously administered in rats at 1e14 vg/kg. Biodistribution and immunogenicity analyses were performed at three months after injection. Results didn't show any significant difference in anti-capsid humoral and cellular immune responses in presence of rAAV9 containing TLR9 DIMS. However, anti-GFP antibody titers were found lower with the engineered vector as compared to the control. Moreover, by RT-qPCR, we determined that rAAV9-TLR9 DIMS was correlated to significantly higher levels of transgene transcripts in skeletal muscles, heart and liver. Finally, the expression of the transgene was maintained until at least 3 months post-injection in the liver of all rats injected with rAAV9 TLR9 DIMS (6/6) *versus* 6/9 in the control group. In conclusion, although there was no impact on anti-capsid immunogenicity, our rAA9 TLR9 DIMS-engineered vector significantly increased the efficiency of gene transfer when injected at systemic high doses and allowed the maintenance of transgene expression. These findings confirm that rAAV engineering with immunomodulatory sequences holds a great promise to suppress or at least modulate rAAV immunogenicity while allowing persisting transgene expression.

1297 Shortening Lentivirus Process Development Timelines Using a Templated Approach

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The field of Lentivirus GMP manufacturing is relatively new, with only a handful of existing therapies on the market today. However, the success of these life-saving therapies is potentiating the development of novel Lentivirus-based therapies and supporting a growing need for Lentivirus GMP manufacture. Further, the timelines for getting regulatory-approved novel therapies to the clinic are compressed due to factors such as patient outlook and the competition. There is a need in the industry to simplify the path to rapid, robust, and compliant manufacture of Lentivirus-based therapeutics. To address the key drivers of Lentivirus manufacturing, we developed a vendor-agnostic, standard manufacturing template containing beginning-to-end guidelines to ensure that the process meets Upstream and Downstream performance metrics, raw materials meet compliance requirements and timelines are predictable. Here we show a case study using this Lentivirus manufacturing template for a model therapeutic. Using DOE-based approaches, we show rapid optimization of Upstream BioProcessing and achieved harvest titers of >5x10^{8}TU/ml. The template permitted rapid and efficient scale-up to 50L scale and generation of high titer material for purification. Using pre-determined template parameters, we were able to obtain efficient recovery of the product with limited iterations. Step recoveries of each step in the Downstream process will be shown. The collective reduction of process development time using this Lentivirus template containing established protocols and compliant raw materials enables rapid manufacture and delivery of life-saving therapies to patients.

1298 Development of a 3-Day DashCAR-T Cell Therapy Against C-Type Lectin-Like Molecule 1 (CLL-1) for Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) characterized by overproduction of immature myeloid cells in the bone marrow is the most common acute leukemia in adults and the second most common leukemia in children. AML standard treatments with chemotherapy, radiation therapy, targeted therapy, and allogeneic hematopoietic stem cell transplantation (alloHSCT) had limited improvements in recent decades as the prognosis of refractory/relapsed AML remains poor and the 5-year survival rate is below 50%. Patients are in urgent need of novel therapeutic strategies. Chimeric Antigen Receptor (CAR) T cells are recognized as one of the most promising breakthrough in the treatment of hematological malignancies that mediate tumor cytotoxicity in a major histocompatibility complex (MHC)-independent manner. Human C-type lectin-like molecule-1 (CLL-1; CLEC12A) is highly expressed on differentiated myeloid cells, AML blasts and leukemic stem cells (LSCs) but not on normal hematopoietic stem cells (HSCs), suggesting that CLL-1 is a compelling target with low off-tumor toxicity for novel AML treatment. A developing CLL-1 DashCAR-T cell in the second generation CAR format, targeted with a human single-chain variable fragment (scFv) against the CLL-1 antigen, was optimized to 3 days in the manufacturing bioprocess, conferring DashCAR-T cells with characteristics of high percentage of CAR population (>80%), high percentages of memory-like phenotypes of T_{scm} and T_{cm} (>70%) and profound effector cytokine production (IFN-γ, granzyme A, perforin and granulysin) as well as specific cytotoxicity against various AML tumor cells both *in vitro* and *in vivo*. In the AML U937-Luc xenograft mouse model, the CLL-1 DashCAR-T cells with CD28 co-stimulatory domain exhibited superior anti-tumor activity than those with 4-1BB domain, and prolonged the survival time with CAR-T cell persistence more than 48 days post CAR-T cell infusion. Furthermore, the xenograft mice receiving as minimum as 3 x10^5 DashCAR-T cells per mouse were able to elicit effective recall responses after tumor rechallenge and survived for more than 60 days. In the aspect of on-target off-tumor toxicity, hematotoxicity assessment revealed that CLL-1 DashCAR-T cell candidates were well tolerated as they exerted modest colony formation inhibition of CD34+ cells derived from normal bone marrow in 5%-23% at E/T = 1 (n=3) and 1%-34% at E/T = 4 (n=2), but had no effect with either E/T ratio on colony formation of CD34+ cells derived from normal peripheral blood. Moreover, autologous CLL-1 DashCAR-T cells were successfully prepared from relapsed/refractory AML patients and were demonstrated to mediate specific cytotoxicity to primary AML blasts and LSCs derived from the same patients. Taken together, the CLL-1 DashCAR-T cell candidates have demonstrated excellent anti-AML

potency and efficacy with minimum safety concerns, and warrant to be further developed preclinically for the treatment of relapsed/refractory AML patients.

1299 Use of Highly Enhanced Autologous Natural Killer Cells for Advanced Alzheimer's and Parkinson's Disease

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Background: Alzheimer's Disease (AD) and Parkinson's Disease (PD) are two very common neurodegenerative diseases with no disease modifying treatments. As more evidence emerges on how misfolded amyloid and alpha-synuclein protein deposits elicit a cascade of autoreactive neuroinflammation and damage, it is clear that removing these proteins by themselves will not fully address the complex process. Natural Killer (NK) cells are an essential part of the innate immune system that have been found to slow progression of amyloid and synuclein protein deposition. NK cells have also been reported to identify and eliminate autoreactive T-cells and damaged neurons via DNAM-1 and NKG2D. But immunosensece has been reported in many patients with neurodegenerative diseases. While NK cells have always been challenging to grow and enhance ex vivo especially when derived from older or heavily pretreated donors, SNK01 is a first-in-kind, autologous non-genetically modified NK cell product with significant increased cytotoxicity and over 90% activating receptor expression that can be consistently produced from any donor. We hypothesize that SNK01 could be consistently produced from any patient with advanced AD or PD, and that it would be safe and potentially beneficial. **Methods:** In preparation for a full Phase I dose escalation study, SNK01 was administered intravenously (IV) to four patients with confirmed advanced AD or PD via compassionate use. **Results:** NK cells were successfully activated and expanded from each patient. Average cytotoxicity was increased over 400% and average activating receptor expression was greater than 90%. There were no adverse events reported in the over 20 total doses given. Each patient had noticeable improvement from their pre-treatment baseline evaluation. **Conclusion:** SNK01 with high cytotoxicity and activating receptor expression can be consistently produced from patients with advanced AD and PD. SNK01 is very safe and may have some clinical activity in AD. This will be expanded into a full Phase I/II trial with a more prolonged dosing schedule.

1300 Engineered Virus-Like Particles Enable High Efficiency of Base Editing in hiPSC-Derived Motor Neurons

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Base editing shows great promise for the treatment of human diseases. For now, adeno-associated viral (AAV) vectors are the primary delivery vehicles for *in vivo* applications. However, the prolonged expression of base editing agents delivered in AAV may cause accumulations of off-target mutagenesis and increased immunogenicity. A prior study reported that the engineered virus-like particles (eVLPs) could efficiently package and deliver base editor or Cas9 ribonucleoproteins (RNPs) with reduced off-target editing. Here, we used eVLPs to deliver base editors into motor neurons derived from human pluripotent stem cells (hiPSCs) of a spinal muscular atrophy (SMA) patient, who carries homozygous for the deletion of exons 7 and 8 in survival motor neuron 1 (SMN1) gene. The paralogous gene *SMN2* is different from *SMN1* by a silent C-to-T transition in exon 7, resulting in the skipping of exon 7 in most *SMN2* transcripts and production of only low levels of SMN protein. Thus, increasing the exon 7 inclusion in *SMN2* has been pursued as a therapeutic approach for SMA. Our results showed that eVLP-delivered base editor and gRNA induced around 40% T-to-C editing of *SMN2* in motor neurons. The editing increased the expression of full length *SMN2* transcripts. SMN protein was also notably detected in the edited motor neurons. This study highlights the therapeutic potential of eVLP-delivered base editor RNPs for SMA treatment.

1301 Non-Viral Gene Therapy Utilizing Transcutaneous Ultrasound-Mediated Gene Delivery

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Transcutaneous ultrasound-mediated gene delivery (UMGD) is a minimally invasive gene delivery strategy utilizing microbubbles and naked DNA. DNA and microbubbles can be delivered through systemic IV administration and ultrasound used to guide DNA into the cells of the organ of interest. A strong body of literature suggests UMGD as an efficient approach for transgenic DNA delivery into various organs including the liver, kidney, pancreas, and heart. However, the lack of systematic data supporting the efficacy, durability, and safety of ultrasound-mediated gene therapy vector delivery prevents its usage for developing genetic medicines. To confirm the feasibility of using UMGD for efficient transgene delivery in the liver, we developed a reporter transgene expression cassette built in various next-generational non-viral gene therapy vectors. Upon ultrasound-mediated delivery of systemically administrated DNA vectors, strong, durable, and dose-dependent transgene expression was detected in rat and mouse livers. Transgene expression was detected as early as 3 hours post treatment suggesting fast kinetics of DNA delivery to hepatocyte nuclei. Subsequent evaluation of safety endpoints one-day post-treatment showed no elevation of ALT, AST, or IL6 levels in blood, confirming safety of ultrasound-mediated transgene delivery to the liver. This study provides a promising strategy for using minimally invasive ultrasound-mediated transgene delivery of next generation gene therapy vectors for developing genetic medicines.

1302 Automated Monitoring, Analytics, and Machine-Learning for Improved Large-Scale AAV Vector Production

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Manufacturing viral vectors for gene therapy requires large-scale, highly controlled production of recombinant adeno-associated virus (AAV) via expensive and time-consuming processes. Therefore, optimizing these processes to maximize vector quality, minimize expense, and avoid manufacturing failures is critical. We sought to improve the overall yield and purity of AAV manufacturing batches by 1) collecting and analyzing manufacturing equipment data during 50L and 500L AAV manufacturing batches, 2) using these data to benchmark against previous batches, and 3) using these data as inputs to a machine-learning (ML) system that forecasts purified AAV output on a per-batch basis. Throughout upstream and downstream processes, we combined data recorded from in-process instrument readings and assay results, processed these data in automated pipelines, and stored these data in a relational database. We then created online dashboards to communicate manufacturing process trends and in-progress status across the organization. Finally, we identified key predictive features from the batch data as inputs for ML models to forecast ultimate yield and quality for in-progress batches. ML Forecasting models were substantially more accurate than naïve estimates of batch output (i.e. the median output of similar runs) over a sample of 50L and 500L manufacturing batches: these forecasts provide valuable insight into the manufacturing process and are potential inputs for prospectively assessing batch results. Additionally, predictive features identified via automated ML feature selection (e.g. cell viability, metabolite levels) provide candidate targets for subsequent manufacturing process improvements. Overall, this system provides a framework to improve AAV manufacturing output by better understanding the root causes of process variance and prospectively identifying likely manufacturing failures through automated analytics and ML-modeling of the manufacturing process.

1303 Autologous Bone Marrow Aspirate Injection as an Adjunct Biological Treatment in Patients Undergoing Lumbar Microdiscectomy

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Introduction Lumbar degenerative disc disease (DDD) is a common pathology in the general population that causes severe disability and societal costs. Sciatica caused by lumbar disc herniation is a common consequence of DDD. Though microdiscectomy has positive outcomes for sciatica patients, the degeneration of the discs progresses post-operatively, often resulting in re-herniation of the disc or the worsening of DDD symptoms such as lower back pain. Therefore, alternative treatment options aimed at disc regeneration are warranted. Injection of autologous bone marrow aspirate (BMA) is a potential biological treatment to treat degenerative disc disease, aid in regeneration of the endogenous disc, and alleviate symptoms related to microdiscectomies. However, data on safety of BMA as an intradiscal injection in patients undergoing microdiscectomy are lacking. Therefore, we aimed to assess the safety and feasibility of injecting BMA at the disc site during lumbar discectomy in patients with sciatica. **Methods** A Phase I clinical trial was designed, which included patients with an indication for microdiscectomy due to a single-level, symptomatic, lumbar disc herniation. In this clinical trial, following tubular decompression of the nerve root, processed BMA (harvested from the iliac crest) is injected at the disc site under fluoroscopic guidance. The rest of the surgical procedure is conformed standard practice.Outcome measures include an extensive physical examination, the numeric rating scale (NRS) for leg pain and back pain, and the Oswestry Disability Index (ODI). These data were collected at baseline, 1 day, 3 months, 6 months, and 12 months after surgery. Additionally, MRI-scans were collected at baseline and 3 and 12 months after surgery. Complications were extensively monitored throughout the trial follow-up. This study was approved by the IRB of Weill Cornell Medicine and written informed consent was collected from all patients prior to inclusion. **Results** Thirty-nine patients have been included. At baseline, patients had a mean NRS of 7.5 and 7.0 for leg and back pain, respectively, and an ODI score of 49.3. At 6 months of follow-up, all patient-reported outcome measures showed statistically significant decreases compared to baseline values. Leg pain decreased to 2.0±1.2, back pain to 2.5±1.7 and the ODI to 27.5±11.8. None of the patients experienced (serious) adverse events or complications related to the surgery or BMA-injection. The MRI T2 mapping showed significantly improved for both disc height signal intensity at 12 months follow up. **Conclusion** Based on the data of the included patients, BMA seems to be a safe and feasible potential treatment adjunct to treat lumbar DDD. Further comparative studies are warranted.

1304 A Phenolic-Molecule Mediated Co-Delivery System for Multiple AAV Vectors

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Introduction Biomedical techniques often require simultaneous delivery of multiple adeno-associated viral (AAV) vectors, especially in CRISPR gene editing and direct reprogramming. However, sample dilution during delivery can hinder the efficacy of delivering all vectors into each target cell, therefore requiring a novel delivery system for robust co-delivery of multiple AAV vectors. **Methods** Biomimetic phenolic molecules have a strong affinity for peptides and biomolecules. Previous research indicated that polyphenolic molecule-treated AAV vectors improved heart transduction *in vivo*. Our hypothesis is that the peptide affinity of phenolic molecules could enhance co-delivery of multiple AAV vectors. To test this hypothesis, we conducted *in vitro*

and *in vivo* experiments and analyzed the co-expression of delivered genes. We also generated a hypoxic ischemic encephalopathy (HIE) mouse model and co-delivered multiple factors to directly reprogram reactive astrocytes into functional neurons, evaluating the recovery of sensory and motor functions. **Results** Our *in vitro* experiments revealed improved co-transduction efficiency of multiple AAV vectors with phenolic molecule treatment, despite localized cell administration. *In vivo* delivery to HI-injured mouse brains showed increased gene expression in reactive astrocytes, as demonstrated by dual staining of GFP and astrocyte markers. The proportion of cells co-transduced with GFP and E2crimson was also higher when treated with phenolic molecules. Lastly, the recovery of sensory and motor functions in mouse models with HI injury through the *in vivo* direct reprogramming of reactive astrocytes into functional neurons will be discussed further. **Conclusion** This study is the first to show improved co-transduction of multiple AAV vectors with phenolic molecules. Future research will focus on optimizing the formulation and exploring its use in CRISPR gene editing and co-expression of intein-split peptides. Overall, the ability to effectively co-transduce multiple vectors has the potential to enhance the therapeutic outcomes of both biomedical and genetic techniques.

1305 IL-2 Preferentially Expands a Less Functional, CD27-Negative CAR T Cell Subset and Compromises Antitumor Potency

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Chimeric antigen receptor (CAR) T cell therapy has been a promising approach to treat some hematological malignancies, but treatment failure and disease relapse are frequently observed across different clinical studies. The characteristics and fitness of CAR T cell products, especially the memory-like phenotypes, have been shown to closely correlate with therapeutic outcomes. *Ex vivo* expansion, usually supplemented with interleukin (IL)-2, is necessary for generating clinical-grade CAR T cell products, but extended expansion can also result in the loss of memory-associated signatures and a compromised effector potency. The cellular dynamics of CAR T cells during *ex vivo* expansion and its impact on antitumor function, however, remain poorly understood. Here we revealed that the expression of CD27, a memory-associated costimulatory molecule, gradually decreased during IL-2-mediated CAR T cell expansion. On the other hand, a differentiated, effector-like CAR T cell subset characterized by the lack of CD27 expression (CD27-), was particularly responsive to IL-2-driven expansion, which became dominant and occupied more than 90% of total CAR T cells after extended culture. This CAR T cell subset was also featured by the exclusive expression of T cell activation markers including CD25 (IL-2 receptor), and the superior responsiveness to IL-2 as demonstrated by a >10 fold proliferative advantage over CD27-expressing (CD27+) CAR T cells. Despite their expansion potency with IL-2, the CD27- CAR T cells were less functional compared to CD27+ cells, especially against large xenograft tumors as well as tumor cells with low antigen expression. The induction of CD27 costimulatory signal requires the interaction between CD27 and its ligand CD70; consistently, the cytotoxicity of CD27+ CAR T cells against CD70-expressing tumor cells was diminished with CD70-blocking antibodies. Meanwhile, CAR-driven tonic signaling and the subsequent acquisition of early-exhaustion signatures, mainly occurred in the CD27- subset. Further, we analyzed 58 CAR T cell products expanded in IL-2, and revealed that CD27 expression was correlated with effector function against recursive tumor cell challenge. The outgrowth of CD27- CAR T cells was suppressed using IL-15-mediated expansion, generating memory-like CAR T cell products and enhanced antitumor efficacy. These results provided the cellular mechanism of IL-2-driven differentiation when expanding therapeutic T cells, revealing the critical need to refine manufacturing procedures and preserve CD27-expressing CAR T cells. This work also suggested the potential of CD27 as a biomarker for the functional quality of CAR T cell products.

1306 LNP-AAV Hybrid Delivery of a DNA Gene Writer System Corrects Severe Ornithine Transcarbamylase Deficiency in Neonatal Mice and Improves AAV Potency in Adult Mouse and NHP Livers

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The use of adeno-associated virus (AAV) for gene transfer and the treatment of genetic diseases has tremendous promise but continues to face challenges in clinical translation such as dose-limiting toxicity and dilution and/or loss mediated by target cell division. One approach to overcome these challenges is to apply methodologies that integrate genes into the genome using integrating viral vectors or by utilizing nuclease-mediated gene transfer. These approaches offer some improvement over AAV's episomal expression in durability of effect but still have undesirable safety risks and/or limited efficiency. To address some of the limitations of previous genetic therapies, we utilize Gene Writer systems - an engineered mobile genetic element - along with its cognate nucleic acid template that is used for integration into the genome. Here, we describe a DNA Gene Writing system that co-delivers the *Sleeping Beauty* (SB) transposable element mRNA via a lipid nanoparticle (LNP) along with an AAV encoding a template for correction of ornithine transcarbamylase deficiency (OTCD) in the *spf^{ash}* neonatal mouse model. The transient mRNA expression of the SB transposable element combined with AAV delivery of the human OTC gene DNA Writer template mediated genomic integration and

led to sustained phenotypic correction of urinary orotic acid levels over 10 weeks. When neonatal mice were treated with the AAV delivered DNA Gene Writer template alone or with an AAV construct designed to express hOTC episomally, the level of hOTC expression when mice reached early adulthood was sub-optimal which resulted in orotic acid levels comparable to untreated mice. Only animals that received the complete DNA Gene Writer system survived after residual murine OTC activity was significantly reduced by silenced expression with an shRNA. Furthermore, application of the LNP-AAV hybrid delivery of the SB DNA Writer system to adult mice or NHPs allows for significant improvement of AAV mediated gene transfer. In adult mice, we demonstrate a nearly 40-fold increase in reporter production delivered with our Gene Writer system compared to an equivalent dose of AAV alone - an effect that cannot be attributed to cell division loss in an adult mouse. In NHP we demonstrate, through use of the non-immunogenic secreted reporter rhesus chorionic gonadotropin β-subunit (rhCG), that the DNA Writer driven stable integration shows 10-fold increased serum concentrations after 13 weeks and did so with enhanced potency, requiring a 5-fold lower AAV dose. Utilization of the SB DNA Gene Writer system could enable dosing at a lower and safer level of AAV as compared to higher levels required for traditional AAV episomal driven expression. Although the current SB DNA Gene Writer system is not site-specific, it is specific in the DNA sequence that is integrated as opposed to random AAV genome integration. These findings of stable and potent delivery of genes mediated by a DNA Gene Writer system exemplify the transformative potential of Gene Writing *in vivo* to treat devastating genetic diseases.

1307 Delivery of PMP22-Targeting shRNA by ARMMs as a Disease-Modifying Therapeutic Approach for Charcot Marie Tooth 1A

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Charcot Marie Tooth type 1A (CMT1A) is a genetic disease caused by duplication of the Peripheral Myelin Protein 22 (PMP22) gene. It is considered the most common inherited demyelinating peripheral neuropathy. Overproduction of the PMP22 protein in Schwann cells leads to myelin loss, axonal damage, and muscle atrophy. Reducing PMP22 expression to non-carrier levels is likely to alter the course of disease. However, to date, there are no disease-modifying therapies for patients due to the absence of an appropriate vehicle that safely deliver molecules, such as short hairpin RNA (shRNA), to suppress PMP22 expression levels in the disease-affected Schwann cells. We engineered ARMMs (ARRDC1 mediated microvesicles), a distinct class of human extracellular vesicles, to actively load short hairpin RNA (shRNA) using ARRDC1 fused to an RNA-binding domain as an active recruitment handle. In non-human primate (NHP) biodistribution studies, we identified Schwann cells as a specific target cell type transfected by ARMMs after intrathecal administration. Using an *in silico* screening approach we prioritized human and NHP PMP22 targeting shRNA sequences, that 1) may yield optimal shRNA knockdown; 2) have limited potential off-target activity; and 3) can target both NHP and human PMP22 transcripts. shRNA candidates loaded into ARMMs were shown to yield functional delivery as evidenced by target engagement in human primary Schwann cells. PMP22 targeting shRNA delivered via ARMMs downregulated PMP22 mRNA in a dose-dependent manner and reduced PMP22 protein levels in Schwann cells. Our data provide evidence for the therapeutic potential of ARMMs loaded with PMP22 shRNA as a disease-modifying strategy for CMT1A.

1308 Synthetic DNA Templates Accelerate Development of mRNA Based Therapeutics and Vaccines

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA for use in gene therapy and vaccines. 4basebio has developed a proprietary, scalable synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. The opDNA™ produced is devoid of any bacterial backbone and the manufacturing process circumvents cumbersome fermentation processes required for plasmid DNA. The process is size and sequence independent, enabling the incorporation of polyA tails >120 bp, and allows for large scale production of linear DNA with high yield and purity in less than a week. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins, and excludes bacterial sequences such as antibiotic resistance genes. Critically, opDNA ™ does not need to be linearised prior to use in an IVT reaction, removing an enzymatic step from mRNA bioprocessing workflows. Here, we compared the synthesis of *in vitro* transcribed (IVT) mRNA using opDNA™ versus linearised plasmid DNA, and achieved significantly higher mRNA yields irrespective of construct size, ranging from 1.8kb reporter genes to 9.5kb samRNA constructs. Proinflammatory cytokine/chemokine levels in primary human PBMCs were comparable to mRNA produced from linearised plasmid, as was 3‘ heterogeneity of the transcripts and levels of dsRNA impurities. 4basebio’s targeted, non-viral delivery platform, Hermes™, allows targeting towards specific cells or tissues of interest, and is suitable for a range of nucleic acid and biological payloads. Using Hermes™ nanoparticles, mRNA reporter constructs were encapsulated and transfected in a range of cells models (HEK293, C2C12, primary human chondrocytes and primary human hepatocytes, PBMCs), and transgene expression was equivalent to mRNA produced from linearised plasmid. Comparable expression was also achieved in mouse models via I.M and I.V routes of administration. We have demonstrated that opDNA™ templates can be used for the production of IVT mRNA. Moreover, the technology could overcome the difficulties associated with complex polyA tails for mRNA constructs, which are inherently difficult to synthesise via bacterial propagation systems. The combination of 4bb mRNA and Hermes™ non-viral delivery platform can greatly accelerate the therapeutic development of gene therapy and vaccine programmes.

1309 Generation of HLA-A2-Specific CAR Engineered CD8 Tregs with Robust *In Vitro* and *In Vivo* Suppressive Activity

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Regulatory T cells (Treg) are key players in peripheral immune tolerance and homeostasis. Thymic derived, endogenous CD4 and CD8 tTreg exhibit regulatory activity across a wide range of animal models and human diseases, including autoimmune disorders, solid organ transplantation and graft vs. host disease (GvHD). Adoptive transfer of gene edited, engineered Treg (EngTreg) has emerged as promising therapeutic approach to induce immune tolerance. We previously described use of homology-directed-repair (HDR)-based gene editing to introduce a ubiquitous promoter into the *FOXP3* locus to generate CD4+ EngTreg with high level FOXP3 expression and stable Treg phenotype and function. In comparison to polyclonal Treg, antigen-specific Treg are vastly more efficient in suppressing autoimmune responses and preventing disease in vivo. As an approach to achieve antigen specificity in organ transplant or GvHD, several groups have introduced chimeric antigen receptors (CARs) derived from anti-HLA-A*02 specific mAbs (A2-CAR) into human tTreg. Compared to polyclonal tTreg, A2-CAR tTreg exhibited more potent suppression of immune responses induced by HLA-A*02 mismatched Teff. In the current study, we expand upon these previous efforts, by utilizing HDR-gene editing and LV delivery to generate either CD4 or CD8 A2-CAR EngTreg. Notably, as part of the HDR editing cassette, we introduced FRB-IL2RB and FKBP-IL2RG fusion proteins (referred to hereafter as chemically inducible signaling complex or CISC), to mediate IL2 like signaling in the presence of a chemical dimerizer, e.g., rapamycin or rapalog. Using this approach, CD4 and CD8 CISC A2-CAR EngTreg were generated and enriched to high purity using rapamycin. Cell products exhibited stable A2-CAR and FOXP3 expression, expression of key Treg markers, and minimal proinflammatory cytokine production but robust TGF-β expression upon stimulation. Like their CD4 counterpart, CD8 A2-CAR EngTreg were able to suppress effector T cell proliferation. Next, we tested whether CD4 or CD8 A2-CAR EngTreg could suppress effector T cells in an allogeneic GvHD NSG model. Our data show that both CD4 and CD8 A2-CAR EngTreg exhibit robust suppressive capabilities against HLA-mismatched allogeneic effector T cells in NSG recipient mice. To compare the effective dose of CD8 vs. CD4 A2-CAR EngTreg, we performed a dose response study that revealed marked efficacy of CD8 A2CAR EngTreg in the allo-GvHD NSG model. Together, our results demonstrate the relevance of the CAR engineering and T cell editing approach to develop antigen-specific CAR/CISC expressing CD8+ EngTreg with potential for clinical application in transplantation, and potentially in other diseases.

1310 A Novel Rat Model for Type A Cystinuria Replicates Pathobiological Features Observed in Patients

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Cystinuria (CYS) is the most common rare kidney stone disease, affecting ~14 in 100,000 individuals globally. It is caused by mutations in either SLC3A1 gene encoding for rBAT (type A cystinuria) or SLC7A9 gene encoding for b0,+AT (type B cystinuria) subunits of a neutral and basic amino acid transporter of Cystine, Ornithine, Lysine and Arginine (COLA) expressed in the kidney proximal tubular epithelial cells and in the intestine. If all COLA have been shown to be increased in urine, only increased cystine levels lead to precipitation of crystals and continuous formation of stones in the urinary tract. The current standards of care aim to prevent calculi formation aim at reducing the concentration of cystine (ie diet), increase its solubility (ie D-penicillamine and mercaptopropionylglycine), or to dissolve stones that have formed over time by lithotripsy (ie ultrasound). However, they have limited efficacy and cause adverse events leading to poor drug compliance and stone recurrence which may lead to kidney damage and even kidney failure in some cases. Patients require repeated ureteroscopy interventions to remove larger stones if they are not eliminated naturally. We engineered a new CYS rat model by deleting exons 1 and 2 of the Slc3a1 gene. This deletion caused complete loss of Slc3a1 mRNA and protein expression in kidney proximal tubules of rats that are homozygote for the KO allele (HOM), with normal expression maintained in heterozygotes (HET). SLC7A9 protein expression was also altered in HOM, with an intracellular localization detected in the proximal tubule compared to apical plasma membrane location in wild-type (WT) and HET. The lack of transporter resulted in a specific increase in urinary COLA in HOM animals. Cystine concentrations were above the solubility level for all HOM rats at 8 weeks of age, the earliest time measured. All KO rats had numerous pathognomonic hexagonal crystals in urine. Bladder stones were detected by μCT scan as early as 9 weeks of age in some of the animals. By contrast, all HET animals had normal urinary COLA levels. Overall, this novel rat model of CYS recapitulates the pathobiological features of the disease observed in humans. We plan to use the CYS rat for pharmacological testing of novel therapeutic interventions for this debilitating disease with high unmet medical need.

1311 Weak Promoter and High Vector Dose to Restore Retinal Function in FAM161A Deficient Ciliopathy (RP28)

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FAM161A encodes for a protein expressed in the photoreceptor connecting cilium (CC). Biallelic loss of function mutations in this gene cause autosomal recessive retinitis pigmentosa-28 (RP28). No treatment exists to cure or slow down disease progression. We developed a gene augmentation approach to restore retinal function and slowdown retinal degeneration. The human *FAM161A* gene produces two major isoforms (Long, L; Short, S) in the retina. We first tested in PN15 *Fam161a* knockout mice AAV2/8 vectors (10^{10} genome copies, GC/uL) coding for IRBP-GRK1-L or -S, or in combination. Single vectors did not improve retinal function, but partially protected the retina. At 3 month post-injection (mpi) of vector combination (L+S), FAM161A was expressed in the CC but also extended to the photoreceptor cell body and behind. ONL was thicker in treated eyes compared to control eyes by around 200%, but retinal function (in scotopic condition) only transiently and modestly improved at 2 mpi (P=0.0055), being similar to control eyes at 3 mpi (n=18 for each group). A lower vector dose (10^{9} GC/uL) did not ameliorate the treatment effect. We thus investigated whether a fine regulation of the protein expression is necessary to recover retinal function and tested with the AAV2/8 vector the endogenous core *FAM161A* promoter with the Crx-binding-region-1 (CBR1) or CBR2. At PN14, the subretinal injections of the CBR2-core-S *hFAM161A* vector (10^{10} GC/uL) provoked a robust gene expression in the CC and ONL at 1 mpi (n=9), while CBR1-core-S (n=6) vector induced a faint expression, only in the CC. A higher injection dose of CBR1-S or L vectors (10^{11} GC/uL) increased the number of FAM161A-positive CCs only, but did not improve retinal activity (n=5 and 7 respectively). However, co-injection of CBR1-core L and S vectors ($0.5x10^{11}$ GC/uL each) induced both homogenous retina labelling in the CC only and functional improvement 3 mpi (n=4, P=0.0004). Retinal integrity was also best improved by administering combinations of vectors. The ONL thickness was similar to WT retina where FAM161A-positive CC density was the highest. We thus propose to use the weak *FAM161A*-core promoter with *CBR1* and high vector doses to treat RP28 with the two isoforms of the FAM161A gene.

1312 Gene Therapy Candidate for Metachromatic Leukodystrophy (MLD): Optimization of HMI-204 Development Candidate

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Metachromatic leukodystrophy (MLD) is an inherited autosomal recessive lysosomal storage disorder (LSD) with a great unmet medical need. This fatal neurodegenerative LSD occurs in three forms: late infantile (prevalence of 1 in 40,000), juvenile, and adult. The first two forms represent the majority of the MLD patients where mortality at 5 years is estimated at 75% and 30%, respectively. MLD is most commonly caused by mutations in the *ARSA* gene and patients suffering from the disease are deficient in arylsulfatase-A (ARSA) enzyme activity. The disease is characterized by accumulation of sulfatides to supraphysiological toxic levels in the peripheral organs and nervous system. In the brain, excess sulfatides lead to the destruction of myelin, a key protective sheath that forms a layer around the nerve fibers that enhances propagation of action potentials. Herein, we report the outcome of the optimization and nomination of a development candidate, HMI-204, for the treatment of MLD. The optimization addressed both biology and manufacturability. The goal was to maintain an intravenous (I.V.) delivery route for HMI-204 to target this disease systemically, while also providing the expression of high levels of ARSA activity in the brain of *Arsa* knockout (KO) mice, predicted to lead to direct motor deficit improvements as demonstrated with the previous lead construct. The team also sought to lower, but not eliminate, ARSA expression in the heart tissue. Following a single I.V. dose of HMI-204, anti-ARSA expression patterns in the brain of adult *Arsa* KO mice remained nearly identical to that of anti-murine Arsa distribution in wild type age-matched littermates, confirming successful crossing of the blood-brain barrier (BBB). A dose-response in ARSA brain activity was achieved in adult and neonate *Arsa* KO mice, reaching normal human levels of expression as measured in normal post-mortem human brain tissue samples. This range covered the expression levels predicted to lead to motor deficit prevention in the MLD mouse model (50-100% of normal human levels) across multiple doses. In the heart of adult *Arsa* KO mice, the biodistribution of anti-ARSA was significantly reduced, while that in the liver remained similar, when compared to the previous lead construct. In neonatal mice, a dose-response in ARSA activity was achieved in heart and liver tissues, as well as in serum, leading to a durable systemic expression for the entire study duration (12 weeks). Lastly, the manufacturing productivity profile of HMI-204 (vg/L) was substantially improved as compared to the previous lead construct. In summary, a single I.V. dose of HMI-204 achieved a broad and sustained systemic biodistribution, including the central nervous system, while lowering expression in heart tissues. Levels of ARSA activity detected in each organ tested reached normal human levels for the corresponding organ, at one or multiple doses. Lastly, the optimization improved both the biological

and manufacturability profile of HMI-204 and these preclinical data continue to support the potential of HMI-204 as an effective gene therapy for the treatment of MLD.

1313 CRISPR-Cas9 *RAG2* Correction via Coding Sequence Replacement to Preserve Endogenous Gene Regulation and Locus Structure

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RAG2-SCID is a primary immunodeficiency caused by mutations in *Recombination-activating gene 2 (RAG2)* which is intimately involved in the process of lymphocyte maturation and function. *Ex-vivo* gene editing of a patient's own hematopoietic stem and progenitor cells (HSPCs) using a combination of CRISPR-Cas9 and rAAV6 vectors could provide a therapeutic alternative to the only current treatment for *RAG2*-SCID, allogeneic hematopoietic stem cell transplantation (HSCT). To do so, we established the most conducive transgene design for efficient homology directed repair (HDR) at the *RAG2* genomic locus while maintaining endogenous regulatory and spatiotemporal elements. As a proof-of-concept single-allelic gene correction, we established a *knock-in/knock-out (KI-KO)* strategy in healthy donor (HD)-derived $CD34^+$ HSPCs via multiplexed HDR. *KI* of our *RAG2* correction donors that replaced the entire endogenous *RAG2* coding sequence (CDS) to preserve endogenous gene regulation and locus architecture led to successful development from $CD34^+$ HSPCs into $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ T cells and promoted the development of highly diverse TRB and TRG repertoires in an *in-vitro* T-cell differentiation (IVTD) platform. We present a method to correct the *RAG2* gene while maintaining *RAG2* expression regulation and locus structure and a workflow to determine the optimal configuration for CRISPR-Cas9/rAAV6 correction of genes with strict spatiotemporal gene regulation.

1314 Directed Differentiation of Gene Edited iPSCs by Small-Molecule Inhibition of a Transgene-Encoded Protein

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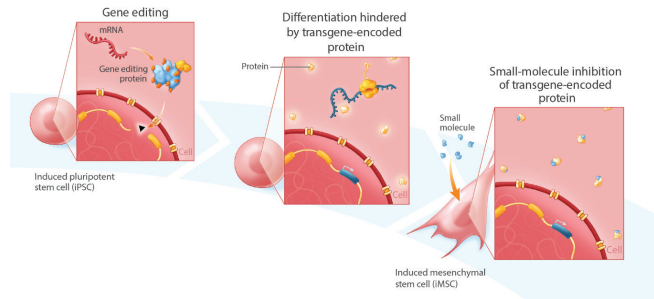
Indoleamine 2,3-dioxygenase 1 (IDO1) is an inducible, heme-containing enzyme that is critically involved in tryptophan catabolism and known to be a prominent immune regulator. Cell therapies with increased IDO1 expression are of high interest for a variety of indications, including autoimmune disorders, inflammatory diseases, transplant recovery, and wound healing. In particular, iPSC-derived mesenchymal stem cells (iMSCs) engineered to overexpress IDO1 may be ideal for suppressing dysregulated immune cells while simultaneously promoting expansion of regulatory T lymphocytes and the M1 (pro-inflammatory) to M2 (anti-inflammatory) polarization of macrophages. Here, we report the development of an iMSC cell line containing an IDO1 transgene under the control of a JeT promoter that was inserted into the AAVS1 safe-harbor locus in mRNA-reprogrammed iPSCs. A clonal population of edited cells (*i.e.* IDO1-iPSCs) was isolated using single-cell sorting. Bi-allelic insertion of the IDO1 transgene was confirmed by amplicon sequencing. The IDO1-iPSCs were then differentiated to IDO1-iMSCs. During differentiation, we found that the IDO1-iPSCs showed an unexpected, cuboidal morphology and noticeably decreased proliferation rates relative to control iPSCs, possibly indicating that IDO1 was interfering with the differentiation process. Two small-molecule IDO1 inhibitors, Epacadostat, which binds to holo (heme-bound) IDO1 and inhibits the enzymatic function of the protein and IDO1-IN-5, which binds to apo (heme-dissociated) IDO1 and inhibits IDO1-dependent cell signaling, were added to the culture for the final five days of differentiation. The addition of both IDO1 inhibitors (each at a 10μM concentration) increased the proliferation of the partly differentiated IDO1-iPSCs (6 h reduction in doubling time) with a population doubling time approaching that of control iMSCs (29 h vs. 27 h). While both inhibitors could enhance proliferation independently, Epacadostat alone had a larger effect on population doubling time than the IDO1-IN-5 alone (6 h vs. 2 h reduction in doubling time), suggesting that the anti-inflammatory enzymatic function of IDO1 may be most responsible for decreased proliferation during differentiation of IDO1-iPSCs. Notably, we also found that when administering both IDO1 inhibitors to non-engineered control iMSCs, the population doubling time increased relative to untreated test cultures (34 h versus 27 h), suggesting a possible minimum IDO1 protein level required for optimal iMSC culture. These results suggest that small-molecule inhibition of transgene-encoded proteins may form a key element of directed differentiation process development for knock-in iPS cell lines, and may be useful for the scale-up manufacturing of IDO1-iMSCs in particular.



1315 PKC Agonists as Small Molecule Inducing Agents for Enhancing Lentiviral Vector Production

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Achieving high upstream lentivector (LV) titres is fundamental to the development and manufacture of a commercially viable gene therapy

product. However, product titres can vary considerably with different therapeutic transgene sequences despite being produced in the same mammalian cell system, so implementing new technologies that are able to recover low titres or improve high titres further can be invaluable. Induction is a key stage of the upstream LV production process that typically involves increasing the expression of LV genes in production cells with the histone deacetylase inhibitor, sodium butyrate. A series of recent screening experiments revealed to us that titres of LV products can be further increased by using an additional class of molecules known as PKC agonists alongside the induction step. Here, we describe how, through optimisation of dosing concentration and timing, LV titres can be enhanced 2- to 9-fold with the non-tumour promoting PKC agonists prostratin and ingenol 3-angelate in a product-specific manner. Importantly, LV produced using PKC agonists have comparable or superior particle-to-infectivity ratios, and residual PKC agonists are removed from the vector product following downstream processing. Furthermore, we demonstrate that PKC agonists act synergistically with an in-house technology based on LV RNA-targeted U1 snRNA to achieve log-fold-increases in titre without detriment to product quality attributes. Having established the benefits of introducing PKC agonists to our platform process and in our packaging/producer cell lines, we provide insight into the mechanisms by which these agonists are acting on production cells and now intend to transfer this technology to GMP manufacturing for commercial products.

1316 Translating Preclinical Efforts into Clinical Gene Therapy Trials: A Systematic Literature Review in the Leukodystrophies

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Background: Leukodystrophies are progressive single gene disorders affecting the white matter of the brain. Several gene therapy trials are emerging to address the unmet need in this patient population. Yet many preclinical efforts stall and do not progress to trials in humans. **Methods:** We performed a comprehensive literature review using the PubMed online electronic database regarding the application of gene-based therapies in leukodystrophies and compared these results to trial listings in www.clinicaltrials.gov, through January 2023. The search was performed using the following keywords: "name of the disease" AND gene therapy. We noted which indications and approaches had received approval by EMA or FDA. Where available, delivery route, targeting cell type, and side effects of treatments were documented. **Results:** Among the approximately 30 disorders characterized as classic leukodystrophies (Vanderver et al, 2015), only 5 have progressed to clinical trials in the field of gene or cell therapy, namely, metachromatic leukodystrophy, X-linked adrenoleukodystrophy, globoid cell leukodystrophy, Canavan disease and Alexander disease. Preclinical studies focusing on varied treatment approaches have been published in another 9 well-characterized disorders, that had not entered human trials yet. While multiple therapeutic approaches have been studied in leukodystrophies including *in vivo* (e.g., adeno-associated virus), *ex vivo* (e.g., lentiviral), and antisense oligonucleotides, only *ex vivo* lentiviral gene therapy for X-linked adrenoleukodystrophy has thus far received EMA or FDA approval. Several challenges emerged in both preclinical and clinical studies: chemical design, proper cell targeting and successful intracellular delivery, intervention window, immunogenicity of the viral vector and associated transgene leading to safety concerns. **Conclusions:** Even in preclinical studies of gene and cell therapy a careful alignment of biological characteristics and target cells specific to each leukodystrophy and trial design recognizing mechanisms relevant to the intervention window is essential. Standardizing monitoring procedures and management of immune responses will need to be addressed before these types of therapies become a widely accepted treatment in the leukodystrophies.

1317 A Robust AAV Production Platform with a Novel 293 Cell Line

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In the past decade, recombinant Adeno-Associated virus (rAAV)-based gene therapies have attracted increasing interest for the treatment of monogenic disorders. Complexity and cost of large-scale rAAV production are still serious obstacles for the development and commercialization of AAV gene therapy products. Several platforms are commonly used to produce rAAV, such as transient triple plasmid transfection of HEK293 cells, recombinant herpes simplex virus (rHSV) infection of HEK293 (or BHK-21) cells, recombinant baculovirus infection of *Spodoptera frugiperda 9* (Sf9) cells, and stable rAAV producer cell lines. Currently, triple transfection of HEK293 cells is the most popular platform due to its simplicity, flexibility and potential for facilitating the vector development from research to clinic trials. To increase the production efficiency and yield, we had spent effort on selection of single cell clones that produced rAVV with high yield. Out of 234 clones, a HEK293 clone, 8F8-4G1, was identified, which produced 3-7 fold more viral vectors compared to parental cell line. The cells were cultured in serum-free medium and grown in suspension, therefore the culture volume could be readily scaled up. In addition, the cell line is stable and can be cultured for multiple passages with high density. After further optimization, with this rAAV production platform, same quantity of the vector was produced using 3-7 fold less culture volume than its parental cell line for large-scale production, resulting consequently in 3-7 fold reduction in the cost. The establishment of the platform with this novel cell line will greatly accelerate the vector research and discovery, and facilitate the cost control in CMC manufacture.

1318 Development and Optimization of Novel Super piggyBac®-Based Hybrid Gene Therapy Approach

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Conventional adeno-associated virus (AAV) therapies have provided an avenue to treat a broad range of diseases. Despite their advantages, AAVs exhibit limited durability when applied early in life. Transgenes

delivered by conventional AAV generally exist as extrachromosomal episomes, and are not propagated during the continuous cell division that occurs early in life. Repeat dosing of current AAV platforms is challenging due to the strong anti-capsid immune response in humans, rendering AAV generally unsuitable for gene therapy treatment in pediatric patients. The super piggyBac® DNA insertion system enables stable integration of a therapeutic transgene into the genome, and comprises two components: 1) a piggyBac transposon, containing the therapeutic gene of interest (GOI) expression cassette, and 2) the super piggyBac transposase (SPB), which is responsible for excising the GOI from the transposon and pasting it into the genome. We sought to develop a super piggyBac hybrid system suitable for liver-directed gene therapy, utilizing existing AAV capsids to deliver the transposon, and a novel liver-directed LNP to deliver an mRNA encoding SPB (LNP-SPB). A broad in vivo screening campaign utilizing design of experiment (DoE) models was performed to identify and optimize biodegradable LNP formulations capable of specific and high-efficiency delivery of mRNA to the mouse liver. Expression of the SPB protein in liver was dose-proportional and transient, peaking between 2-4 hours and declining to undetectable levels within several days. Tandem mass spectroscopy studies demonstrated clearance of the novel ionizable lipid from the liver within several hours following intravenous administration. The LNP was well-tolerated, with no clinically significant changes in body weight, complement activation, or clinical chemistry at doses up to 2 mg/kg. Evaluation of the LNP in adult cynomolgus demonstrated a similar liver-tropic biodistribution as in mice, and no clinically meaningful tolerability signals were observed at all doses evaluated. We evaluated three liver-tropic AAV capsids for delivery of a piggyBac transposon: AAV8, AAV9, and KP1. Each capsid was able to be successfully manufactured at small scale with a transposon comprising a therapeutic human gene and the piggyBac inverted terminal repeats. Co-administration of each capsid with the LNP-SPB to neonatal (day=1 of life) mice resulted in substantial (20-60%) transduction of hepatocytes, which was maintained into adulthood. Between 1-4 integrated copies of the GOI per diploid genome were detected by ddPCR in the animals treated with AAV + LNP-SPB. Administration of each capsid alone (without the SPB LNP) to neonatal mice resulted in minimal (<3%) hepatocytes expressing the GOI, presumably due to dilution of the episomal transgene during liver growth. Co-administration of the LNP-SPB with AAV did not result in any detectable tolerability concerns. In a mouse model of the urea cycle disorder ornithine transcarbamylase deficiency (OTCD), we observed that disease resolution could be achieved with significantly lower doses of AAV when combined with the LNP-SPB relative to AAV alone. These data demonstrate the versatility of the piggyBac hybrid platform, which can be deployed effectively in a variety of AAV capsids. Further, we demonstrate the advantage of this system for achieving durable transgene expression when administered early in life and at lower total AAV doses.

1319 Rationally Designed Cardiotropic AAV Capsid Demonstrates Targeted Cellular Distribution in Cardiomyocytes

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Congestive heart failure (CHF) is a progressive disease that is characterized by the heart's inability to pump adequately to meet the body's demands. This widespread condition has a critical unmet medical need. Recent advances in understanding the molecular basis of myocardial dysfunction have resulted in AAV gene-therapy (GT) clinical trials for heart failure. Cardiac tissue analysis from earlier clinical trials that failed to show efficacy revealed poor vector transduction. A novel cardiotropic and liver de-targeted capsid, AAV2i8, was rationally designed to overcome poor transduction in cardiac tissue. Our clinical trial for the treatment of non-ischemic cardiomyopathy uses the AAV2i8 capsid to deliver a constitutively active protein phosphatase 1 inhibitor 1 (I-1c). We previously reported that molecular evaluation of vector performance from left ventricular biopsy tissue of a subject enrolled in our trial revealed high vector transduction and mRNA expression. We also observed restoration of depressed S16 phosphorylation of phospholamban, which is associated with abnormal intracellular calcium handling, a key cellular abnormality in CHF. The molecular studies performed previously provide a confirmation of our vector efficacy (ASGCT 2022). Here we report development of cellular distribution methods to study our vector behavior at the cellular level. Probes targeting the CMV promoter region and I-1c transgene region were designed for RNAscope, a recently developed in situ hybridization method allowing detection of cellular and subcellular localization of the vector genome DNA and I-1c mRNA in the mouse heart tissue and human biopsy. Wheat germ agglutinin (WGA) was used to label cell membrane and cardiomyocytes were distinguished from endothelial cells that were co-stained with Isolectin B4 (IB4) in mouse heart tissue. Method optimization was performed in control human and mouse hearts. Mice were injected with saline or AAV2i8 I-1c vector through tail vein and sacrificed at 14 days post injection. Heart tissues were harvested, snap frozen on dry ice and cryosectioned at a thickness of 10μm. Heart sections were subjected to fluorescent RNAscope assay followed by WGA and IB4 staining. Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. We found that transduced vector genomes and I-1c mRNA were predominantly observed in cardiomyocytes of mouse heart tissue, demonstrating that our vector efficiently transduces its target cells within the myocardium. Examination of control human heart tissue by RNAscope and cellular and subcellular marker staining showed that I-1c was not detectible in the absence of AAV2i8 I-1c vector administration. Here we present cellular distribution of our vector in the human left ventricular biopsy. These data underscore the importance of obtaining molecular quantitation as well as cellular distribution of vector performance for novel capsids in human tissues, and also support the validity of our GT approach towards the treatment of heart failure.

1320 A Lipid Nanoparticle Toolkit to Correct Cystic Fibrosis in Airway Stem Cells

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Aim: Cystic fibrosis (CF) is a monogenic disorder resulting from mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that leads to chronic lung infections & respiratory failure. Effective delivery of biomolecules to self-renewing airway basal stem cells (ABSCs) for gene correction in the CF airway has been especially difficult because of their protected location within the epithelium. We report the design and testing of a gene therapy strategy that utilizes lipid nanoparticles (LNPs) configured to: i) access ABSCs by penetrating the dehydrated mucus layer in the CF airway *via* surfactant-incorporating liposomes engineered for epithelial disruption (LNP 1) and ii) package & deliver *CFTR*-directed CRISPR/Cas9 gene editing payloads to exposed ABSCs (LNP 2). Methods: We utilized electroporation of Cas9 ribonucleoprotein complexes into immortalized human bronchial epithelial cells (16HBE14o-) to screen various single-guide RNA constructs (sgRNAs) for their ability to edit within the endogenous *CFTR* 5' untranslated region (UTR) and integrate a reporter cassette encoding green fluorescent protein (GFP). Integration was measured *via* digital-droplet PCR. LNP formulations were synthesized *via* microfluidic mixing or rapid ethanol dilution and characterized by dynamic light scattering. Primary bronchial cells were cultured at an air-liquid interface (ALI) to mimic the epithelial structure of the human airway. The ALIs were exposed to LNP 1 suspensions, and ABSC exposure and epithelial repair was assessed *via* confocal microscopy. Flow cytometry was used to quantify transfection of 16HBE14o- cells treated with LNP 2 formulations loaded with mRNA transcripts encoding GFP. Particle formulations found to produce transfection efficiencies of >50% were applied to deliver *CFTR* gene editing cargoes. Editing efficiencies in 16HBE14o- cells were evaluated *via* tracking of insertion-deletions by decomposition. Results: We identified a sgRNA with >95% cutting efficiency, no detected off-target editing, and integration rates of 10-15% at the *CFTR* 5'UTR. We incorporated a primary amine with a 6-carbon linker to the 5' end of double-stranded DNA donor cassettes *via* PCR amplification to increase integration by 2-fold over traditional donors. We identified LNP 1 formulations containing the surfactant polidocanol capable of transiently exposing ABSCs in ALI cultures. Compared to polidocanol alone, LNP 1 induced similar degrees of ABSC exposure with more extensive epithelial repair 24h after treatment. We evaluated several LNP 2 formulations integrating different ionizable lipids for RNA delivery. Optimal LNP configurations contained the ionizable lipid SM-102, which, when packaged with mRNA transcripts encoding GFP, achieved transfection rates *ca.* 70% in 16HBE14o- cells and preserved *ca.* 80% cell viability. Editing efficiencies of *ca.* 10% were observed with particles prepared with SM-102 packaged with mRNA transcripts encoding Cas9 and a sgRNA targeting *CFTR*, compared to <3% with particles containing other ionizable lipids. Conclusion: We have designed gene editing biomolecules to effectively target *CFTR,* and demonstrated encapsulation & functional delivery of these reagents to airway epithelial cells. We have further developed strategies to access target ABSCs with minimal injury. The tools and methods developed here serve as building blocks for establishing CF gene therapy solutions poised to accelerate progress toward our collective goal to find a permanent cure for CF.

1321 Production, Purification and Characterization of Engineered ARRDC1-Mediated Microvesicles Using a Scalable Manufacturing Platform

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Clinical evaluation of extracellular vesicle-based therapies requires development of robust production and purification processes to enable scalable and consistent GMP manufacturing. Here we report purification and characterization of a class of engineered extracellular vesicles called ARMMs (ARRDC1-mediated microvesicles) using a transient transfection-based or a stable producer cell line production process. For transient transfection, HEK5B8 suspension cells were transfected with ARMMs loading constructs using polyethyleneimine. We also engineered HEK5B8 cells to stably produce ARMMs loaded with GFP as a cost-efficient and consistent source of engineered ARMMs that overcomes the challenges of variability and reduction in viable cell density often observed following transient transfection. Material generated using either ARMMs production approach were evaluated using several analytical methods to quantify size, loading efficiency and homogeneity. The methods used included nanoparticle tracking analysis, western blotting, ELISA and nanoFCM single particle analysis. Data supported using a stable producer cell line for scalable production of engineered ARMMs. HEK5B8 stable ARMMs producers were scaled in a 3L batch process and ARMMs purification was carried out using an optimized industrial process. Lastly, we tested uptake of ARMMs *in vitro*, and *in vivo* in mice. Intravenous administration led to robust uptake of ARMMs in the spleen and liver. These data were in line with lab scale generation and evaluation of ARMMs, supporting their future manufacturability for evaluation in clinical trials.

1322 Comparison between Droplet Digital PCR and Quantitative PCR for Adeno-Associated Virus Infectious Titer Assay

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Recombinant adeno-associated virus (rAAV) has been utilized successfully for *in vivo* gene delivery for the treatment of a variety of human diseases. There is a critical need for the development of robust analytical methods to sustain the growth of rAAV gene therapy products and ensure safe and appropriate therapeutic dosages selected for treatment. $TCID_{50}$ assay (50% Tissue Culture Infectious

Dose) is an *in vitro* cell-based method widely used to determine AAV infectivity and this assay is historically viewed as a challenge due to its high variability. Currently, quantitative real-time PCR (qPCR) serves as the end-point method to detect the amount of replicated viral genome after infection. Here, we evaluate how well the droplet digital PCR (ddPCR) can be adapted into $TCID_{50}$ assay as an end-point detection method. By direct comparison between qPCR and ddPCR read-out, we observed a trend of improved inter-assay precision when the ddPCR method is utilized. Particularly, we offer improvements of $TCID_{50}$ infectious titer assay with: (1) higher precision and sensitivity by adapting ddPCR as an end-point method without standard curve preparation; (2) identification of an important second "set threshold" value in infectivity scoring; and (3) application of statistical analysis to identify the acceptance range of infectious titer values. Taken together, we provide an optimized $TCID_{50}$ method with improved accuracy and robustness that is important for rAAV infectious titer testing during process development and manufacturing.

1323 *In Situ* Production of Extracellular Vesicles

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Introduction: Nucleic acid-based medicines have opened a new avenue in drug discovery, allowing targeting, expression, or silencing of essentially any gene. However, the major challenge for their therapeutic utility remains functional encapsulation to ensure their stability and efficient delivery. In the past decade, extracellular vesicles (EVs) have emerged as a next-generation drug delivery system owing to their intrinsic ability to efficiently convey macromolecules. As native nanoparticles, EVs benefit from immune tolerance as well as the ability to cross biological barriers to reach distant organs such as the brain and intestine. Importantly, they can be engineered to carry macromolecules of interest. Despite enormous progress in the development of EVs for therapeutic purposes that brought them from pre-clinical studies to various clinical trials, successful transition to clinical applications remains challenging. Current approaches in above-mentioned studies and clinical trials use exogenously produced EVs, manufacturing of which at large scale requires investment in process development capabilities and notably, exert faster plasma clearance, requiring reoccurring administrations. To address this, we have developed a novel platform, "in situ EV production", where we have utilized gene therapy modalities to engineer cells *in vivo* to harness endogenously secreted EVs for delivery of biotherapeutics to hard-to-reach organs. **Methods and results:** Building upon advanced EV engineering strategies to load macromolecules, previously developed in our lab, we tagged an EV sorting domain with Nanoluciferase (NLuc) for highly sensitive tracking of in situ produced EVs. The EV engineering transgenes were administered either as a plasmid DNA via high-pressure injection or as mRNA encapsulated into lipid nanoparticles (LNPs). Importantly, both these delivery strategies have tropism to the liver and in part to the spleen. Consequently, produced EVs are primarily of hepatic origin, which is reflected in the high Nluc signal in liver. Importantly, NLuc tagged EVs showed a body-wide distribution to all major organs, including hard to reach tissues such as the CNS. In other words, we achieved sustained release of engineered EVs in circulation and showed a drastic increase in pharmacokinetic profile over exogenously administered EVs. This approach, however, is only applicable for transient production of EVs due to the relatively short expression of LNP formulated transgenes. To achieve long-term in situ production of EVs, we administered NLuc tagged EV transgenes as a ssDNA incorporated into adeno-associated viruses (AAVs). Over a period of 4 weeks, we observed increasing NLuc activity in serum, proving sustained release of in situ produced Nluc EVs to the bloodstream. At endpoint, we detected NLuc EVs throughout the body, including pancreas, brain and intestine. For assessment of functional protein delivery, we used Cre recombinase loaded EVs in an Ai9 mouse model, which induces expression of the fluorescent reporter TdTomato upon successful recombination. The EV engineered transgene was administered in AAVs and functional transfer of Cre-loaded EVs was assessed by flow cytometry of single cell suspensions and immunohistochemistry of murine tissues. TdTomato positive cell populations were detected in all major organs analyzed, demonstrating functionality of *in situ* produced Cre-loaded EVs. Furthermore, using in situ approach, our preliminary data on therapeutic versatility showed promising results in murine model of Chron´s disease. In summary, we have developed platform for endogenous production of engineered EVs using liver as a biofactory. Considering its applicability for short and long-term EV release, as well as advanced EV engineering approaches, we are confident that this innovative and versatile platform could be repurposed for the delivery of any desired biotherapeutic cargo.

1324 A Simple and Efficient Feeder-Free Workflow for Expanding and Genome Editing Blood Natural Killer Cells

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Natural killer (NK) cells are innate lymphocyte effectors that kill cancerous or virus-infected cells and can modulate immune responses by secreting proinflammatory cytokines. NK cells show immense promise for immunotherapies targeting challenging cancers such as solid tumors. The development of such cellular therapies requires the ability to genetically engineer and generate clinically relevant numbers of functional NK cells. We have developed a serum-, xeno-, and feeder-free culture system that enables the expansion and genetic manipulation of peripheral blood NK cells. Fresh human NK cells were obtained from peripheral blood leukapheresis using EasySep™ magnetic isolation and were either cultured immediately or cryopreserved in CryoStor® CS10. Fresh or thawed NK cells were cultured in ImmunoCult™ NK Cell Expansion Medium in plates coated with ImmunoCult™ NK Cell Expansion Coating Material. NK cells were replated on days 7 and 10, and harvested on day 14 prior to phenotyping and functional assessment. In some experiments, NK cells were cultured until day 21 to obtain higher cell yields. The average frequency of CD56+CD3- NK cells on day 14 was 90 ± 0.7%, with 75 ± 2.1% being CD16+. After 14 days of culture, the NK cells expanded on average 66 ± 9.8-fold (mean ± SEM, n = 78), and increased to 461 ± 129-fold (n = 13) when cultured for 21 days. Cryopreserved NK cells also achieved significant

expansion after 14 days in culture, although lower than freshly isolated NK cells, with an average expansion of 30 ± 6.2-fold (n = 25). Genetic engineering is an important tool utilized in NK cell research. To ensure compatibility with CRISPR-Cas9 genome editing, NK cells were cultured for 3 - 4 days on coated plates prior to ribonucleoprotein (RNP) delivery in ImmunoCult™ NK Cell Expansion Medium. Using electroporation, ArciTect™ CRISPR-Cas9 RNP was delivered into activated NK cells, after which the cells were returned to culture. Guide RNA sequences were designed to target either CD45 or TIGIT as representative cell surface markers expressed by NK cells. Gene editing efficiencies of 91 ± 3% and 88 ± 3% of CD45 and TIGIT were observed 4 days post-electroporation, respectively (n = 4). To assess functionality, the expanded NK cells were co-cultured with K562 cells to assess natural cytotoxicity, or co-cultured with SK-BR-3 breast adenocarcinoma cells in an antibody-dependent cellular cytotoxicity (ADCC) assay. For the ADCC assay, SK-BR-3 cells were treated with anti-HER2 antibody for 30 minutes and washed prior to assessment. On average, intracellular expression of IFN-γ was observed in 49 ± 3.7% (n = 15) of K562-stimulated NK cells, and in 32 ± 4.1% (n = 7) of NK cells cultured with antibody-treated SK-BR-3 cells. Additionally, intracellular expression of TNF-α was observed in 43 ± 3.7% (n = 8) of K562-stimulated NK cells, and in 28 ± 4.1% (n = 7) of NK cells cultured with antibody-treated SK-BR-3 cells. Stimulated NK cells also expressed surface CD107a, a marker of degranulation (73 ± 5.2% for K562 co-culture, [n = 15], and 63 ± 3.3% for ADCC assay [n = 7]). Finally, NK cell target killing was measured in co-cultures. Target cells were treated with a substrate that, when cleaved by activated caspase-3/7, results in the release of a fluorescent dye. At an effector:target ratio of 1:1, an average of 50 ± 2.6% (n = 11) of K562 cells, and an average of 60 ± 2.4% (n = 7) of antibody-treated SK-BR-3 cells were killed. The work presented here highlights the suitability of our NK cell expansion system to edit and expand functional NK cells to aid in the study and development of cellular immunotherapies.

1325 Generation and Persistent Genetic Modification of Virus- and Factor-Free Human iPSCs into Kidney Organoids Using S/MAR DNA Vectors

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The generation of isogenic or autologous cells for cell- or organoid-based disease modelling and therapies relies on the efficient reprogramming of patient-derived cells. This is often achieved using integrating viral vectors, which carry a danger of insertional mutagenesis and oncogenesis. Alternatively, the most common non-integrative reprogramming technologies still require viral components (EBNA1/oriP, Sendai) or multiple transfection steps (RNA). These methods however cause concerns; cells reprogrammed with viral components have elevated markers of cellular damage and immune signalling, and repeat transfections increase both the cost and the risks of non-compliance with quality standards for clinical use. Therefore, to reduce these risks and improve the potential of the cells for clinical application, we have developed a method to reprogram primary human fibroblasts using fully non-viral DNA vectors, which require only a single transfection. Our vectors are based on the use of a Scaffold Matrix Associated Region (S/MAR), which is a sequence of human origin and drives autonomous episomal replication of the vector in the host cell nucleus. This allows the sustained expression of factors required for reprogramming, with loss of the vector after epigenetic silencing in the late stages of reprogramming, leading to true vector-free, factor-free human induced pluripotent stem cells (hiPSCs). We have shown that hiPSCs reprogrammed with our S/MAR vectors are morphologically and phenotypically indistinct from EBNA/oriP-derived hiPSCs, and express pluripotent stem cell markers (Tra-160, Nanog, Oct3/4, Lin28). We can also use our S/MAR DNA vectors to genetically modify hiPSCs in a stable and heritable way. Importantly, the vectors are maintained episomally at low copy number and do not damage the cells, as they retain their pluripotent capacity. The expression of the vector transgene in hiPSCs is stable over time (>150 days) in the absence of selection, and is not affected by cryogenic cell storage. In addition to this, transgene expression is stable throughout all stages of differentiation into target cell types: we have shown transgene persistence in tri-lineage differentiation of S/MAR-modified hiPSCs into germ layers as well as directed differentiation into embryoid bodies and kidney organoids. We have modified hiPSCs with S/MAR DNA vectors expressing green fluorescent protein (GFP) and erythropoietin (EPO). EPO is an endocrine factor produced by the kidneys in response to hypoxia, which stimulates erythropoiesis. EPO production is disturbed in patients with chronic kidney disease anemia or kidney transplant injury. We differentiated GFP/EPO-modified hiPSCs into kidney organoids and demonstrated that they remained fully functional, forming glomerular and tubular structures marked by podocytes and proximal and distal tubular cells, while retaining high expression levels of both GFP and EPO throughout the differentiation process. Organoids were then subcutaneously transplanted into mice and retained their structure as well as EPO expression, providing a proof-of-concept for the S/MAR DNA vector-based modification of hiPSCs for functional studies of translational applications. This study gives us the possibility to generate and modify patient-derived iPSCs using our S/MAR DNA vectors, and to differentiate these into target cell types and organoids for further study or implantation as disease models and treatments.

1327 Triple-Modality Therapy with a Dual-Targeting CD133/PD-L1 CAR-T and Anti-PD-1 Plus Radiotherapy Maximize Antitumor Response in Solid Tumor

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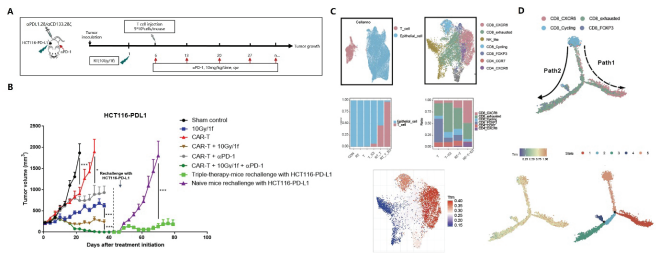
Application of CAR-T therapies in solid tumors is largely hindered by tumor microenvironment (TME), which hampered CAR-T infiltration and transduced inhibitory signals to T cells. Introducing novel CAR-T modules and designing combinational strategies are necessary to overcome these obstacles. We co-expressed an αPDL1. CD28 chimeric receptor with a second generation CD133 CAR-T in a bicistronic vector. Remarkably, the αPDL1.CD28 structure **triggered the CD3ζ signaling in cis by forming clusters with CD133 CAR via CD28 dimerization**. Based on this feature, a novel dual-targeting CD133/PD-L1 CAR-T (αPDL1.28/αCD133.28ζ) was designed, which effectively eliminated either PD-L1+ or CD133+ cancer cells while reducing vector CD3ζ redundancy. **Dual CAR-T showed improved cytotoxic function against CD133+ tumor cells both in vitro and in vivo, regardless of basal PD-L1 expression**. Mechanistically, CD133+ tumor cells upregulated PD-L1 when engaging αCD133.28ζ CAR module to suppress CAR-T function, which instead increased their vulnerability to dual CD133/PD-L1 targeting CAR-T cells. The dual targeting CD133/PD-L1 CAR-T successfully hijacked the PD-L1/PD-1 axis and turned the immune escape mechanism of tumor cells into their weakness. Next, we combined our CAR-T cells with radiotherapy to further enhance the efficacy. In tumor-bearing mice, synergistic anti-tumor efficacy of mice treated with hyperfractionated radiation (10Gy/1f) and dual targeting CAR-T cells was further observed. The **triple-modality therapy with CAR T cells and anti-PD-1 plus radiotherapy maximized antitumor responses and induced complete cures in the tumor-bearing mice.**



Graphic 1. A) Treatment scheme of triple-modality therapy; B) Tumor growth in each treatment group (including rechallenge); C) Single-cell transcriptome analysis of CAR-T cells in TME, a unique CXCR6+ CD8+ CAR-T subset was identified in RT-T & RT-T-ICI group, which posed a high Trm signature; D) Pseudotiming analysis revealed that in RT+T & RT+T+ICI group, CAR-Trm was derived from cycling CAR-T cells.

Single-cell transcriptional profiling of the CAR-T cells in TME revealed that radiation induced a unique tissue-resident memory CAR-T subset that highly expressed CXCR6. In vitro co-culture of radiated tumor cells and CAR-T cells also induced a higher proportion of CAR-Trm cells. Therefore, we conclude that radiation promoted the differentiation of CAR-Trm to prolong persistent anti-tumor toxicity. **In summary**, our study for the first time introduced a novel CAR-T design with two CAR structures sharing a single CD3ζ through a membrane CD28 dimerization. Dual-targeting CD133/PD-L1 CAR-T effectively enhanced anti-tumor toxicity by overcoming the inhibitory signals from TME. More importantly, we demonstrated a rationale and efficacy to combine the novel CAR-T and anti-PD-1 with radiotherapy in solid tumor, which was a paradigm-shifting finding and was implicational for future clinical use. Our study was fully fleshed out and ready to be published.

1328 Single-Nanoparticle Characterization of Co-Encapsulated Multi-Nucleic Acid Species in Lipid Nanoparticles

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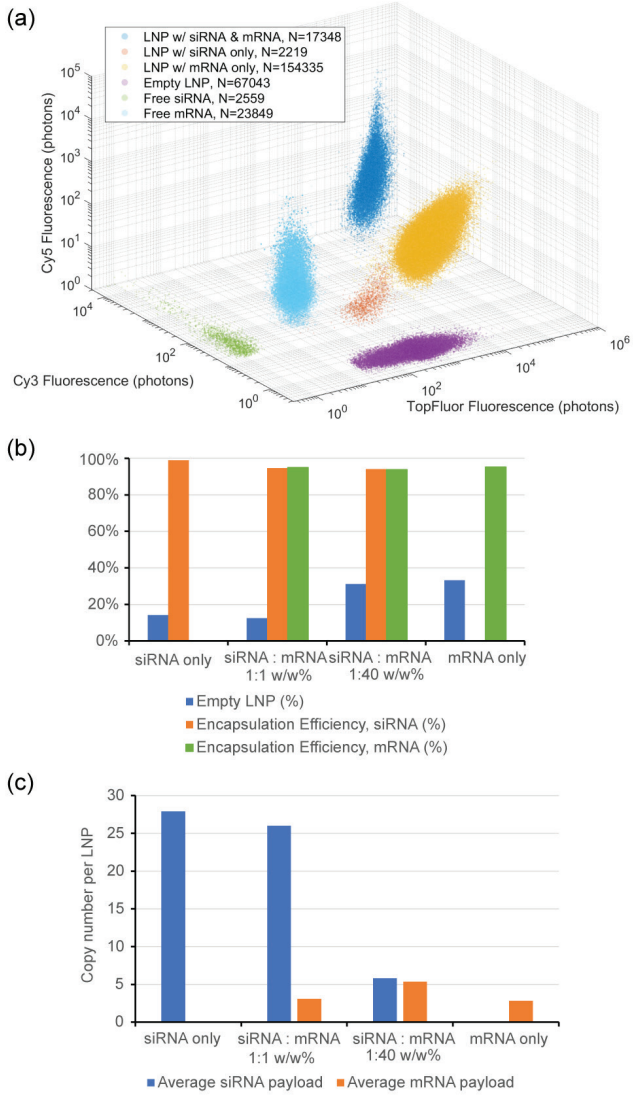
Introduction: Co-encapsulation of multi-nucleic acid species in lipid nanoparticles is an important approach of co-delivery strategies for gene editing (guide RNA and mRNA) and transcriptional modulation (mRNAs and siRNAs) [1]. In such systems, both cargos need to enter the same cell to achieve the intended functional outcomes; therefore, high co-encapsulation efficiency and tunability of the loading level of each cargo are of interest. However, current characterization techniques have limited capability to examine these features on the single-nanoparticle and populational levels, and this lack of tools is hampering the optimization efforts of the formulations. Here, we apply a recently developed technique[2] to elucidate the payload content and distribution of the RNAs in a benchmark formulation for siRNA and mRNA co-encapsulation. The findings of this study will contribute to a comprehensive understanding of the co-delivery effect. **Methods**: We used Cylindrical Illumination Confocal Spectroscopy (CICS) to examine the LNPs with Cy3-siRNAs, TopFluor-PC lipids, and Cy5-mRNAs. Fluorescence coincidence analysis was performed to classify the LNP subpopulations, and deconvolution analysis was applied to the resolve siRNA and mRNA payload in LNPs. A benchmark formulation was prepared with the dosage of ionizable lipid DLin-MC3-DMA, cholesterol, helper lipid DSPC and DMG-PEG2000 at a molar ratio of 50: 38.5: 10: 1.5. The siRNA and mRNA were mixed at a weight ratio of 1:1 or 1:40, in addition to the mRNA only and siRNA only conditions. The LNPs were formulated by rapid mixing in a T-junction at pH 4.0 and dialyzed against PBS at pH 7.4. **Results**: Using the CICS technique, we screened the LNPs at single nanoparticle resolution and identified six subpopulations including (a) LNPs with both siRNAs and mRNAs, (b) LNPs with siRNAs only, (c) LNPs with mRNAs only, (d) empty LNPs, (e) free siRNAs, and (f) free mRNAs (Fig. 1a). LNPs with siRNA: mRNA at 1:1 weight ratio had the same level of empty LNPs as LNPs with only siRNAs (Fig. 1b, 1st and 2nd group, blue bar), while LNPs with siRNA: mRNA at 1:40 weight ratio had a similar level of empty LNPs as LNPs made with only mRNAs (Fig. 1b, 3rd and 4th group, blue bar). All four formulations showed >94% encapsulation efficiency for both RNAs (Fig. 1c). The payload analysis of RNAs co-encapsulated in LNPs showed that: at 1:1 weight ratio, LNPs could accommodate around the same total number of siRNAs and mRNAs (mean = 26.0, 3.1, respectively) as the LNPs formulated with only

siRNAs (mean = 27.9) or mRNAs (mean = 2.8). Nonetheless, increasing the mRNA:siRNA weight ratio to 40:1 elevated mean mRNAs contents in the co-encapsulated LNPs to 5.4 from 2.8 for LNPs with only mRNA, which suggests that co-encapsulated siRNA may increase the loading capacity of mRNA in LNPs.



Conclusions: A new single particle analysis method is developed to characterize the co-encapsulation ability of two RNA species in lipid nanoparticles. Our findings of the distribution of the two payloads in co-encapsulated LNPs compared to LNPs formulated with single RNA species suggest that the payload capacity and co-loading efficiency may be influenced by the ratio of siRNA and mRNA. These findings in the complex assembly behavior of the RNA co-delivery in LNPs could benefit the designs of LNPs for multi-nucleic acid cargos. **References.** (1) *Nat. Nanotechnol.* **2022**, *17* (7), 777-787. (2) *Nat. Commun.* **2022**, *13* (1), 5561.

1329 Elucidating Mechanisms of *Cis* and *Trans* Cleavage to Engineer Improved Cas12a Variants

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CRISPR-Cas9 gene editing technology has proven great potential for gene therapy in pre-clinical models. However outstanding issues such as on-target and off target effects, or accessibility to T-rich targets remain to be solved. CRISPR-Cas12a (formerly Cpf1) enzyme provides a complementary approach to Cas9 on T-rich regions in the genome. Commonly used Cas12a orthologues are less efficient than Cas9 for target cleavage, although editing results in fewer translocations at the target site. In addition to target cleavage, Cas12a possesses a collateral and indiscriminate '*trans*' cleavage activity, useful for molecular diagnostic applications but not considered ideal for gene editing. Our extensive study of the target cleavage preferences of three well described Cas12a orthologues have allowed the precise dissection and identify key elements in the CRISPR-Cas12a system for target cleavage, and collateral cleavage reactions. We harnessed these mechanistic insights to engineer improved variants of Cas12a; with slower off-target cleavage for more precise gene editing and modulation of the collateral cleavage activity. Overall, these engineered Cas12a variants will enable safe precision editing in T-rich genomic regions.

1330 Current Approaches and Considerations for Viral Clearance for Cell & Gene Therapy

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The assurance of viral safety in Cell and Gene Therapy (CGT) products poses a unique challenge as the inclusion of a viral vector is a key component of both *in vivo* and *ex vivo* gene therapies. Although viral clearance strategies for general biological drug manufacture and vaccine production will be applicable to these products, there will be unique challenges and considerations for cell and gene therapy manufacture. Therefore, members of the BioPhorum's Cell & Gene Therapy validation work stream have provided a publication that is intended to indicate in a single location all relevant existing guidance and advice on viral clearance for viral vectors with emphasis on Adeno-associated viral (AAV) vectors. Additionally, the team aims to highlight the unique considerations and potential options to resolve the identified challenges and recommend a 'gold standard' requirement for viral clearance for manufacture of cell and gene therapies. The aim of this publication is to help companies identify the challenges, current guidance and potential solutions for viral clearance within the cell and gene therapy field.

1331 Rapid High-Throughput Screening and Uninduced Recovery of AAV Producer Cell Lines

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Adeno-associated virus (AAV) vectors are a powerful tool for gene therapy applications due to their ability to efficiently and specifically deliver therapeutic genes to target cells. However, current

standard manufacturing methods are not capable of producing titers necessary to treat large patient populations with systemic doses due to challenges in scaling and cost of goods (COGs). Stable producer cell lines (PCLs) are emerging to overcome these challenges, since they can be cultured at scales similar to antibody therapeutic production and do not require transfection with costly plasmids prior to harvest. Some research demonstrates additional benefits such as increased titer and better product quality, such as a higher capsid full/empty ratio, in the crude product compared to the conventional triple-plasmid, transient transfection production method. Despite these advantages, PCLs account for a small proportion of clinical-stage manufacturing techniques due to the additional development time required to engineer a PCL platform, create monoclonal lines, and assess clone productivity. This work describes a microfluidic approach to sort, assay, and recover uninduced clones from a polyclonal PCL pool of thousands in under two weeks to address the need for shorter development time of stable AAV producer cells.

1333 Elucidation of Minimal Adenoviral Helper DNA Sequences Essential for Recombinant Adeno-Associated Virus Production

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Recombinant adeno-associated viral vector (rAAV) is increasingly being used for gene therapy applications because of its non-pathogenic nature, superior safety profile and ability to transduce both dividing and non-dividing cells. The increased usage has led to a considerable surge in demand for clinical grade rAAV vectors. Triple plasmid transient transfection of HEK293 cells is the current state of the art method used to meet the demand for clinical grade rAAV vectors. This method uses three plasmids constituting of: Rep-Cap, adenoviral helper genes and ITR-flanked transgene for producing rAAV vectors. Previous research has identified adenoviral E1A, E1B, E2A, E4orf6 and VA RNA genes essential for rAAV production. Although the essential genes have been identified, the minimal helper DNA sequence sufficient for rAAV production has not been clearly established. The commonly used helper gene plasmid (referred as DeltaF6) still contains several adenoviral complete or partial coding sequences that do not play any direct role in rAAV production. Considering the GMP perspective, accurately defining genetic sequences is extremely significant and is still lacking in case of adenoviral helper genes. Although the adverse effects of the presence of these sequences is not known, they may have an impact on production quality and yield. By selective elimination process, we constructed adenoviral helper gene plasmids that are devoid of these extra sequences and our preliminary data suggests that these newly constructed plasmids positively impact rAAV production while reducing genetic footprint by ~3kb. Furthermore, our research is extending towards identification of minimal sequences (regulatory and protein-coding) of E2A, E4orf6 and VA RNA genes, essential for rAAV production. In conclusion, our work lays the foundation for accurate definition of minimal genetic components necessary for rAAV production while having a positive impact on yield and possibly in future incurring an indirect influence on the gene therapy cost.

1334 Targeted CRISPR/Cas9 Screen Identifies Superior HEK293 Cell Lines for AAV9 Production

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There are two dominant AAV production platforms: transient transfection of mammalian cells and baculovirus infection of Sf9 insect cells. Academic and industry-led studies have shown Sf9 cells produce higher titers of rAAV, but it comes at a cost of vector quality and potency for some rAAV serotypes. Few studies have reported on systematically engineering cell systems to enhance both viral production capacity and potency. To address this, we used clonal isolation and high-throughput screening to develop a proprietary clonal suspension-adapted HEK293 cell line (AC001.230) that produces AAV9 with increased potency compared to HEK 293F cells. Through a targeted CRISPR/Cas9 screen, we identified 3 classes of targets that significantly increased AAV9 production compared to our wild-type clonal cell line. From this work, we have developed two independent suspension-adapted knockout cell lines (AC003 and AC010) that show greater than 2-fold improvement in AAV9 production capacity. We have confirmed these findings using capsid titer and vector genome quantification using a reporter construct. Studies are underway to evaluate the potency of these cell lines for production of FDA-approved therapeutic transgenes using multiple AAV serotypes and in a large-scale bioreactor system.

1335 SKG0106 is a Promising Gene Therapy Vector with Great Therapeutic Potential for the Treatment of Patients with Neovascular Age-Related Macular Atrophy

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Introduction: Neovascular age-related macular atrophy (nAMD) is a progressive disease that leads to severe vision loss in the elderly population. Vascular endothelial growth factor (VEGF) is overexpressed in the eyes of nAMD patients and interacts with VEGFR2, resulting in abnormal blood vessel proliferation and leakage. Recombinant Anti-VEGF proteins that developed for nAMD need to be injected every 4-8 weeks for the rest of patients' lives. We have developed a proprietary AAV vector SKG0106 carrying a transgene encoding an Anti-VEGF protein Nb24. By a single intravitreal (IVT) injection, the continuously expressed Nb24 targets ocular overexpressed VEGF in the lesion area, stopping or slowing down the neovasculature growth and leakage. We tested the binding affinity of Nb24 to human rhVEGF-A and the inhibitory potency of Nb24 to block the interaction of human rhVEGF-A with VEGFR2, with Lucentis, Beovu and Eylea as controls. The therapeutic efficacy and biodistribution of SKG0106 and a reference vector were also compared in NHP CNV model. **Methods:** Binding affinity of Nb24 protein, Eylea, Beovu and Lucentis with rhVEGF-A protein were conducted by SPR or ELISA. A cell-based study was conducted to compare the inhibitory potency of Nb24 and Eylea to block the interaction of rhVEGF-A with VEGFR2 by using VEGFR2 / NFAT Reporter - HEK293

Recombinant Cell Line. In the pharmacological study using NHP CNV model, vehicle, Eylea, SKG0106 and a reference vector were administered via a single IVT injection. The efficacy of Nb24 was assessed on blocking the leakage of the fluorescein in the eyes, and the animals were sacrificed at 43 days. Tissue and blood samples were collected to evaluate the biodistribution and expression of Nb24. **Results:** The KD values of Nb24 and Eylea binding to rhVEGF-A protein were 1.98e-13M and 1.68e-13M, respectively, by SPR assay. In the functional ELISA assay, the EC50 values of Nb24 and BEOVU to rhVEGF-A were 0.21 nM and 0.29 nM, respectively. The IC50 values of Nb24 and Lucentis in blocking the binding of human VEGF-A with human VEGFR2 were 25.56 nM and 62.08 nM, respectively, by competitive ELISA assay. Nb24 and Eylea also inhibited the luciferase expression induced by human VEGF-A in VEGR2/NFAT-HEK293 reporter cells, and the IC50 values of Nb24 and EYLEA were 0.24 nM and 0.32 nM, respectively. In the pharmacological study, SKG0106 completely blocked the fluorescein leakage in the treated NHP eyes and was better tolerated than the reference vector. In addition, the biodistribution and expression levels of SKG0106 and Nb24 were meticulously assessed. The results indicated that the vector and the protein were primarily located in ocular tissues and fluids, with no detection in peripheral tissues. **Discussion and Conclusions:** Nb24 has a high affinity to VEGF-A. The protein potently blocked the interaction of human VEGF-A with its receptor VEGFR2, therefore inhibiting the signal transduction of VEGF-A, implicating that it has a potential to block the VEGF-A mediated neovascularization in the eyes of nAMD patients. Furthermore, SKG0106, an AAV vector delivering Nb24, robustly inhibited abnormal fluorescein leakage via IVT injection in the eyes of NHP CNV model (also in rabbit RNV model, data not shown), indicating that the vector can function effectively in vivo. Quantification of Nb24 by ELISA revealed that the protein was mainly located in the ocular space and was not detectable in most peripheral tissues, suggesting that the vector did not affect peripheral tissues. Importantly, SKG0106 was well-tolerated in the study and others (poster submitted separately). In conclusion, SKG0106 is a potent efficacious vector for the treatment of nAMD and has an excellent safety profile. It has the potential to be the next generation drug for the treatment of nAMD.

1336 Treatment of Recessive Dystrophic Epidermolysis Bullosa by Transplantation of Col7A1 Transgenic Tissue-Engineered Skin Graft

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[1]GeCell Therapeutics, Shanghai, China,[2]Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China

Recessive dystrophic epidermolysis bullosa (RDEB), caused by loss of function mutations in the COL7A1 gene encoding type VII collagen, is a devastating, often fatal, inherited blistering disorder lacking curative therapies. Epidermal stem cells/keratinocytes derived from skin tissue can be expanded, engineered with functional COL7A1 gene and further differentiated into the whole epidermis in vitro, which hold great promise for the treatment of RDEB. Here, we generated a gene-corrected tissue-engineered skin graft from RDEB epidermal stem cells via non-viral vector-Piggy/Bac transposon under the serum-free and chemical-defined culture media. The resultant COL7A1 transgenic tissue-engineered skin graft could integrate into mouse skin for a long period, restoring the COL7A1 expression without tumorigenicity risk. Moreover, clinical data from Investigator-Initiated trial (IIT) showed good efficacy and tolerance on RDEB patient indicated with partial restoration of the COL7A1 expression, good wound healing and no adverse effects after autologousCOL7A1 transgenic tissue-engineered skin graft transplantation. Collectively, our COL7A1 transgenic tissue-engineered skin grafts are promising for RDEB treatment in the future clinical study.

1337 Process is Product: High Yield NK Cell Production Processes Reshape Product Quality Profile

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Robust manufacturing production of natural killer (NK) cells has been challenging in allogeneic NK cell-based therapy. Here, we compared the impact of cytokines on NK cell expansion by developing recombinant K562 feeder cell lines expressing membrane-bound cytokines, mIL15, mIL21, and 41BBL, individually or in combination. We found that 41BBL played a dominant role in promoting up to 500,000-fold of NK cell expansion after a 21-day culture process without inducing exhaustion. However, 41BBL stimulation reduced the overall cytotoxic activity of NK cells when combined with mIL15 and/or mIL21. Additionally, long-term stimulation with mIL15 and/or mIL21, but not 41BBL, increased CD56 expression and the CD56bright population, which is unexpectedly correlated with NK cell cytotoxicity. By conducting single-cell sequencing, we identified distinct subpopulations of NK cells induced by different cytokines, including an adaptive-like CD56brightCD16- CD49a+ subset induced by mIL15. Through gene expression analysis, we found that different cytokines modulated signaling pathways and target genes involved in cell cycle, senescence, self-renewal, migration, and maturation in different ways. Together, our study demonstrates that cytokine signaling pathways play distinct roles in NK cell expansion and differentiation, which sheds light on NK cell process designs to improve productivity and product quality.

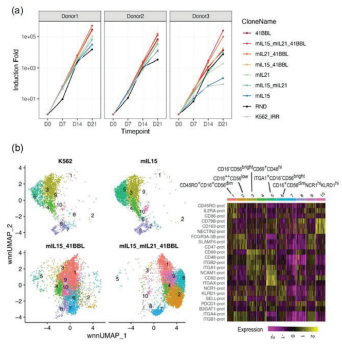

Figure 1: Developing robust NK cell manufacturing processes with high yield and desired differentiation subsets

(a) NK cell expansion fold induction (y-axis in log10 scale) from three donors with indicated process conditions.
(b) Single cell analysis, TotalSeqC panel measured RNA expression and surface protein expression, reveals 10 NK cell clusters. Different process conditions drive NK cells to follow distinct differentiation trajectories, leading to divergence in cellular composition that affect the bioactivities of the cellular products.

1338 Enhancing SaCas9 Target Specificity by Rational Directed Mutagenesis

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Innovative development of the CRISPR/Cas9 system is beginning to make therapeutic gene editing a viable biomedical objective. *Staphylococcus aureus* Cas9 (SaCas9) is sufficiently compact to be packaged into AAV and is therefore commonly used for *in vivo* genome editing. However, fidelity remains a critical barrier to clinical translation. While high-fidelity SpCas9 variants have been reported, few SaCas9 variants have eliminated off-target editing, leaving cleavage error-prone repair of non-target genomic sites a critical issue to be resolved for this ortholog. Here, we developed a traffic light reporter system that examines on- and off-target editing efficiency and used it to screen rationally engineered SaCas9 variants. Using this system, we identified SaCas9 variants (variants harbouring mutations at position Y256, R314, Q414 and R654) with enhanced-accuracy and single base mismatch discrimination while maintaining robust on-target activity comparable to WT-SaCas9. Sanger and next generation sequencing allowed us to confirm dramatically reduced off-target editing (2- to 255-fold improvements) of our variants compared to WT-SaCas9 at three genomic loci. Finally, when delivered by an AAV vector our variants showed no detectable off-target activity when targeting SHLD1 locus in HeLa cells compared with WT-SaCas9. Thus, our variants could be used for genome editing applications where high precision and fidelity are required.

1339 The Unrealised Utility of Unique Molecular Identifiers ('Barcodes') as Quantifiable Reporter Elements - Demonstrations in the Study of ITR Integrity in the rAAV Vector System

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The need to clonally amplify AAV ITR sequences in one form or another underlies the production of all recombinant AAV vectors on the globe. These sequences are centrally involved in numerous intracellular process needed to generate and direct nonviral DNA sequences into their capsid delivery vehicles. It is therefore plainly a problem that plasmid materials used to rAAV vectors are typically heterogenous in one or both ITR loci due to poor stability in prokaryotic culture. ITR sequences have a recalcitrant reputation among rAAV researchers and we have yet to benefit from sustained efforts to understand their heterogeneity and its implications for rAAV vector manufacturing and performance. As is true for many salient challenges, the variability within and between the myriad of active rAAV production systems, and the consequences of stepwise losses and gains, call for an especially robust methodological infrastructure if durable scientific progress is to be achieved. Scientists in rAAV research have been responsible for ground-breaking methodological innovations exploiting massively parallel sequencing technology in the analysis of nucleic acid copies with mixed origins, made distinguishable and countable by short, localised sequence variations often referred to as 'barcodes', most prominently in capsid biodistribution studies. The core analytical principles are conceptually versatile and outstandingly well suited to the task of making robust and precise quantitative measurements of like nucleic acid subpopulations. Its unique quantification mechanism makes it an exceptional complement to dPCR or qPCR for molecular biologists writ large and in many contexts a superior choice. In the present study, this technique was used to track rAAV vector genomes derived from subclonal isolates of an rAAV plasmid with various deletions in the 3'ITR sequence. Barcodes were used to track these genomes and their products through multiple stages of vector synthesis in adherent HEK293 cultures, as well as in *in vivo* transduction of murine liver and skeletal muscle over an eight-week period. The study had a dual goal of showcasing the versatility and metrological capabilities of the barcode analysis strategy, as well as initiating a sustained and rigorous enquiry into the question of rAAV ITR integrity. The strategy resulted in quantitative consistency between samples replicated wholly from the point of transfection comparable with qPCR replicates of single vector samples. Reducing the fraction of intact ITRs in the transfection did not result in a clear decrease in total vector yield, though this particular study was not designed for the detection of incremental differences. Astoundingly clear by contrast were linearly predictable changes to subpopulation composition reflecting ITR-specific variations in process efficiency within a transfected culture. Intriguingly, the greatest variations in stable *in vivo* transduction were observed between vectors derived from intact plasmids, with the effects of whole deletions less prominent in comparison. These results form one component of a larger doctoral dissertation that will be submitted for examination by the time abstracts enter review.

1340 Development of UX055 AAV Gene Therapy for Cyclin-Dependent Kinase-Like 5 (CDKL5) Deficiency Disorder (CDD), a Rare Neurological Disorder

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Ultragenyx is developing UX055, a new adeno-associated viral vector-based gene therapy product for the potential treatment of Cyclin-dependent Kinase-like 5 (CDKL5) Deficiency Disorder (CDD). UX055 is a non-replicating recombinant adeno-associated virus serotype 9 (rAAV9) gene therapy containing a single-stranded DNA vector with the human CDKL5 gene under control of a neuron-specific transgene promoter. This gene therapy approach involves the transfer of a normal copy of the human CDKL5 gene to neurons, thereby enabling expression of functional CDKL5 protein for a prolonged period following a single injection via intracisternal magna (ICM) delivery, to directly address the underlying cause of the disease. Development of AAV gene therapies for rare neurological disease in pediatrics

requires a significant focus on safety and efficacy considerations in the development of the GMP manufacturing process. In developing potential gene therapy treatments for rare genetic disorders targeted to the central nervous system (CNS), careful considerations on route of administration, formulation selection, drug product concentration, dosing strategy, including dosing volume and endotoxin limits, and delivery device must all be assessed to ensure patient safety is maintained at an achievable efficacious dose. Ultragenyx has incorporated these key considerations for CNS administration into the UX055 Clinical Development Plan to achieve a safe, robust, and scalable GMP manufacturing process, that enables rapid generation of clinical material at 2000L scale to enable dosing of children with CDD.

1341 Visualizing Non-Encapsidated DNA in the Adeno-Associated Virus (AAV) Samples by Immuno-Localization Transmission Electron Microscopy (Immuno-TEM)

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Adeno-associated viruses (AAV) are most often used vectors for in vivo gene therapy. Their advantages are stability, size, as they are small enough to cross the blood-brain barrier, and generally mild immune response. In the process of preparing a clinically pure solution of the viral vectors, the detection of present impurities is very important. Among the most commonly present nucleic acid impurities are the remains of the plasmids used in transfection, and host cell DNA. Molecular assays are usually used for quantification of those impurities, however, it is still not totally clear, whether all detected impurities are encapsidated or not. We have developed and optimized an immuno-localization transmission electron microscopy (immuno-TEM) for visualization of non-encapsidated DNA in the preparation of AAV samples. Immuno-localization was performed by anti-dsDNA antibodies (ABCAM) and IgG with bound gold grains of diameter 6 nm. The method was used on the AAV samples and we were able to show the presence of non-encapsidated DNA, mostly bound to the surface of the viral particles. Some of this residual DNA remained present on the surface of the capsids even after nuclease treatment. The presence of bound, nuclease resistant, non-encapsidated residual DNA presents a challenge for purification and might pose a concern for patients.

1342 Lipid Nanoparticle Library Towards Development of Next Generation Genomic Medicines

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Ionizable amino lipids are a major constituent of the lipid nanoparticles for delivering nucleic acid therapeutics (e.g., DLin-MC3-DMA in ONPATTRO®, ALC-0315 in Comirnaty®, SM-102 in Spikevax®). Scarcity of lipids that are suitable for cell therapy, vaccination, and gene therapies continue to be a problem in advancing many potential diagnostic/therapeutic/vaccine candidates to the clinic. Herein, we describe the development of novel ionizable lipids to be used as functional excipients for designing vehicles for nucleic acid therapeutics/vaccines in vivo or ex vivo use in cell therapy applications. We first studied the transfection efficiency (TE) of LNP-based mRNA formulations of these ionizable lipid candidates in primary human T cells and established a workflow for engineering of primary immune T cells. We then adapted this workflow towards bioengineering of CAR constructs to T cells towards non-viral CAR T therapy. Lipids were also tested in rodents for vaccine applications using self-amplifying RNA (saRNA) encoding various antigens. We have then evaluated various ionizable lipid candidates and their biodistribution along with the mRNA/DNA translation exploration using various LNP compositions. Further, using ionizable lipids from the library, we have shown gene editing of various targets in rodents. We believe that these studies will pave the path to the advancement in nucleic acid based therapeutics and vaccines, or cell & gene therapy agents for early diagnosis and detection of cancer, and for targeted genomic medicines towards cancer treatment and diagnosis.

1343 HSPC Gene Therapy Using Transcriptional Targeting to Macrophages Ameliorates Very Early Onset IBD Due to IL10R Deficiency

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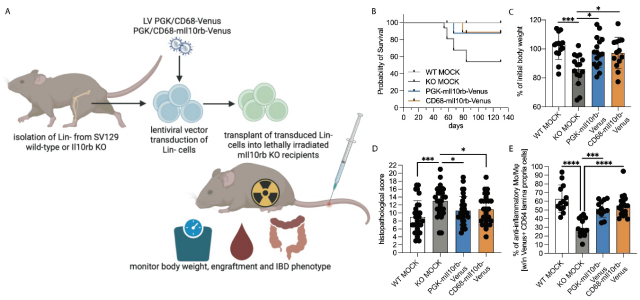
Mutations in the IL-10 gene or its receptor lead to life-threatening Very Early Onset-Inflammatory Bowel Disease (VEO-IBD). Allogeneic stem cell transplant is the only curative option, leading to prolonged disease remission and survival. Because gene therapy represents an attractive therapeutic alternative, we evaluated the efficacy of two different lentiviral vector-based gene therapy approaches for IL-10 receptor beta chain (*mIl10rb*) deficiency. The first one utilizes the PGK promoter for ubiquitous transgene expression. The second approach exploits the CD68 promoter for myeloid cell-targeted transgene expression with maximum activity in mature macrophages. Gene transfer into murine *Il10rb* KO lineage negative (Lin-) HSPC restores responsiveness to IL-10 in vitro using both vectors, as measured by phosphorylation of STAT3 in differentiated macrophages. Mice treated with gene therapy or healthy donor cells (A) showed increased survival (B) and regained body weight within 20-60 days from treatment returning to more than 90% of their weight before irradiation (C), indicating amelioration of the disease, while mock-treated mice progressively lost weight dropping below 80% of their initial weight. Histopathological analysis of colon sections revealed improved tissue architecture after

gene therapy, including reduced inflammatory cell infiltration, reduced hyperplasia and improved recovery of secretory goblet cells with pathological scores significantly reduced to 10.6±3.6 and 10.8±3.7 in PGK and CD68-treated animals respectively compared to mock-treated being at 13.31±3.4 (D). On the cellular level, macrophage cell populations in the lamina propria are believed to be central to regulating immune responses. After gene therapy, we observed increased anti-inflammatory macrophages from 29.15±10.34% in mock-treated to 51.43±9.69% and 54.95±12.69% (E) and reduced proportions of inflammatory macrophages from 20.54±9.13% in mock-treated to 3.9±3.67% and 4±3.1% in lamina propria extracts in PGK- and CD68- gene therapy groups respectively. While both vectors performed comparably regarding therapeutic efficacy, we unexpectedly observed selective loss of vector copy numbers in the PGK- group. In competitive transplantation assays, we confirmed the progressive loss of cells transduced cells over time. While gene marking remained relatively stable in HSC/MPP, a pronounced 2-fold reduction of gene marking was seen in committed progenitors and mature cell lineages. This competitive disadvantage could be bypassed by expressing *mIl10rb* under the control of the CD68 promoter, which led to stable gene marking. This study demonstrates the therapeutic efficacy of a lentiviral-based HSPC gene therapy approach in a relevant mouse model of VEO-IBD, comparing constitutive and myeloid-specific promoters and demonstrating that myeloid-specificity is superior for the permanent correction of clinical features.



1344 Gene Therapy for GM1 Gangliosidosis Mediated by AAV Vector Carrying BBB-Penetrable Enzyme

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[Background] GM1 gangliosidosis (GM1) is a lysosomal storage disease in which mutations in the GLB1 gene result in reduced or absent lysosomal β-galactosidase (βgal) activity. βgal deficiency leads to accumulation of the substrate GM1-ganglioside in the central nervous system (CNS) resulting in progressive neurological decline. There is currently no effective treatment for the disease and in the infantile form is fatal within a few years of onset. Therefore, there is an unmet need for the development of new treatments.Although several gene therapy trials using AAV have been reported for the treatment of GM1, the effect on the CNS has been limited due to the blood-brain barrier (BBB). Recently, we reported an anti-transferrin receptor antibody (TfR) fused enzyme that could cross the BBB and formulated as Pabinafusp Alfa for MPS II enzyme replacement therapy (ERT). Here we report the combination of the TfR system and AAV mediated gene therapy to treat GM1 murine models. [Methods] AAV9s were designed to express the βgal (AAV-βgal) or the TfR-βgal (AAV-TfR-βgal) genes under the control of the mouse albumin promoter to allow the liver-specific expression.Ten-week-old male GM1 mice were intravenously injected with each of the AAVs at $1x10^{12}$ (low dose) and $5x10^{12}$ (high dose) vg/kg. The enzyme activity in serum was measured over time after administration of each virus. Twenty-three weeks after the treatment, quantification of the enzyme activity and the amount of GM1-ganglioside were performed in peripheral organs and CNS. The therapeutic effect was also evaluated by several behavioral experiments. [Results and Discussion] Biochemical quantification showed that the serum βgal activity of each AAV-treated mice was approximately 10-fold higher than that of the wild-type control at 6 weeks post-infection, and this activity was maintained throughout the experiment. In the liver and spleen, βgal activity was higher in the AAV-treated groups than in the wild type. In contrast, in the CNS, such as the cerebrum, βgal enzyme activity increased only in the TfR-βgal group, and was approximately 50% of that of the wild type in the high-dose group. GM1-ganglioside accumulation in the cerebrum, cerebellum, and hippocampus was reduced in a dose dependent manner only in the TfR-βgal treated groups. The amount of GM1-ganglioside in the group treated with low dose of TfR-βgal downed in half and with high doses of TfR-βgal was found to be at the same level as in wild-type mice. Furthermore, TfR-βgal treated mice exhibited improvements in some

of the behavioral experiments such as open field test. These results show that intravenous administration of AAV- TfR-βgal is an effective approach for the treatment of GM1 at such a low viral dose.

1345 Advanced Characterization of DNA Molecules in rAAV Vector and Plasmid DNA Manufacturing Preparations by Next Generation Sequencing

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Adeno-Associated Virus (AAV) based gene therapies have demonstrated clinical success, resulting in a growing demand for clinical- and commercial-grade manufacturing. To support this success, manufacturing highly pure AAV vector preparations at large scale and quality is a focus area for many groups. Characterization of rAAV vector and plasmid DNA for both research grade (non-GMP) and GMP-grade has relied on the gold standard of chain-terminating dideoxynucleotide (Sanger) sequencing for identity testing. However, Sanger sequencing has technical limitations and drawbacks that limit its use for comprehensive sequencing-based characterization of gene therapy products. Therefore, alternative methods that can more comprehensively screen products while increasing throughput rates and decreasing the time to results are needed for continued growth and quality oversight of drug products. Newer sequencing technologies, such as sequencing by synthesis (SBS), have become the *de facto* standard for many clinical and research applications. The massively parallel capacity exceeds that of Sanger sequencing and can provide highly accurate data. More recently, single-molecule sequencing has been commercialized by other groups that allows for full-length vector and plasmid sequencing. In our study comparing these methods, we have assessed sequencing-based methods for analyzing both plasmids and recombinant AAV gene therapy products. We compared the strengths, weaknesses, and overall utility of three sequencing platforms (from Illumina, PacBio, and ONT) for the characterization of rAAV vectors. We developed methods for optimizing sequencing workflows and analyzed vectors sequenced on all three platforms to compare their utility in various quality control tests, such as sequence identity confirmation and contaminant analysis. We then designed a comprehensive plasmid characterization and manufacturing workflow, allowing for the confirmation of plasmid identity and genome integrity, contaminant analysis. The workflow allows us to assess and ensure plasmid quality throughout the production lifecycle, leading to higher-quality products. We show that, by leveraging the strengths of sequencing-based analytics, we can accurately and unambiguously screen and characterize plasmids and vectors, both for research-grade production and GMP-grade drug products. This improves quality and production standards, resulting in high-quality and safe products with faster turnaround times.

1346 RNA-Templated Editing Using Type V CRISPR Effectors

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The programmable nature of RNA-guided CRISPR nucleases stimulated a genome editing revolution, and RNA-templated systems promise to enable precise genomic repair. In this work, we present new systems for genome-primed Reverse Transcriptase-editing (RT-editing) that operate with a CRISPR Type V nuclease. Our implementation with the Type V system demonstrates precision editing within human cells mediated by optimized versions of Type V CRISPR RT-editing systems. The compact nature of the Type V effectors and their gRNAs confer advantages regarding delivery and manufacture relative to existing precision editing systems. RT-editing using a Type V nuclease has shown significant precision editing in therapeutically relevant cell types including T-cells and iPSCs, an editing profile that can be well suited to addressing specific classes of human disease. This work highlights an initial proof of concept in expanding RNA-templated editing to the broad biochemical and mechanistic diversity of Type V CRISPR systems, potentially enabling additional translational potential of RT editors.

1347 Development of AAV Mediated Gene Therapy Approach for Usher Syndrome Type 2

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Purpose: Usher syndrome type 2 (USH2) is the leading cause of deaf blindness with autosomal recessive inheritance. Several pathogenic mutations in *USH2A* gene have been reported to cause USH2 and non-syndromic retinitis pigmentosa (RP). Majority of *USH2A* mutations are frameshift or non-sense mutations leading to loss of function. Development of genetic therapies for USH2 have been mainly hampered due to the large size of the gene (15.6kb). USH2A protein contains several repetitive domains, and it is plausible that some of these are dispensable for its function. AAV-mediated gene supplementation is the most effective and feasible therapeutic approach making it as an expedited path for the treatment of inherited retinal degenerations (IRDs). With the aim to miniaturize *USH2A* that is amenable for AAV mediated gene therapy, we have assembled and validated *USH2A* minigene constructs for their ability to rescue ciliary phenotype in vitro. Using split intein protein trans-splicing system, we have showed the reconstitution of minigene in vitro with the potential for clinical translation in future. **Methods:** To generate *USH2A* minigenes, we used the coding sequence of the short non-repetitive region of *USH2A* as a backbone and the repetitive motifs as building blocks to generate minigenes with motif deletion in different length/region. We have evaluated the functional efficacy of these clonal minigene constructs using an *Ush2a* null Ock-1 cell line and identified a functional *USH2A* minigene (minigene-4). Immunofluorescence was performed to analyze the ciliary morphology and number in *Ush2a* null cells. Expression, localization, and reconstitution of full length minigene

was analyzed by cellular fractionation and western blotting. **Results:** Our data confirmed the expression and cellular localization of minigene-4 in periciliary region. Furthermore, our results indicate that in vitro supplementation of minigene-4 resulted in ciliary rescue in *Ush2a* null cells. Additionally, we have showed the reconstitution of minigene-4 in vitro using split intein mediated protein transplicing approach. **Conclusion:** Our results indicate the identification of functional *USH2A* minigene which can rescue ciliary defects in vitro. Our data indicate the reconstitution of minigene-4 for the utility and suitability for future clinical applications. These findings highlight the therapeutic potential of minigene-4 for treatment of USH2.

1348 Exon Skipping and Dystrophin Production with ENTR-601-45, a Novel EEV-Conjugated, Exon 45 Skip Amenable PMO in Preclinical Models of DMD

Ziqing Leo Qian, Nelsa L. Estrella, Amy N. Hicks, Ajay Kumar, Avery Guan, Sara Blake, Scarlett Cheon, Xiang Li, Anushree Pathak, Mahboubeh Kheirabadi, Patrick G. Dougherty, Wenlong Lian, Nanjun Liu, Ningguo Gao, Daniel Wang, Matthew Streeter, Mohanraj Dhanabal, Mahasweta Girgenrath, Natarajan Sethuraman

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Duchenne muscular dystrophy (DMD) is a rare, X-linked neuromuscular disorder caused by mutations in the *DMD* gene resulting in a lack of functional dystrophin. Antisense phosphorodiamidate morpholino oligomer (PMO)-mediated exon skipping therapies have been approved for several mutations but produce only a very modest amount of dystrophin in skeletal and cardiac muscle, likely due to poor distribution to target tissues and a limited ability to escape the target cell endosome. To overcome these limitations, we designed a family of proprietary cyclic cell-penetrating peptides that form the core of our Endosomal Escape Vehicle (EEV™) technology. This EEV-PMO approach demonstrated exon skipping and dystrophin production in the D2-*mdx* mouse model of DMD. Furthermore, a single intravenous dose of ENTR-601-44 (a *DMD* exon 44 splice modulating EEV-PMO) showed durable exon skipping in skeletal and cardiac muscle in human dystrophin-expressing (hDMD) mice and non-human primates for at least 12 weeks post-dose. To further address unmet needs in the treatment of DMD, we developed an EEV-conjugated PMO for the treatment of patients amenable to exon 45 skipping therapy (ENTR-601-45). Treatment of DMD patient-derived skeletal and cardiac muscle cells with ENTR-601-45 resulted in robust exon skipping and dystrophin production. Additionally, a single IV dose of ENTR-601-45 resulted in high levels of exon skipping in hDMD mouse skeletal and cardiac muscle after 1 week. These results demonstrate the therapeutic potential of ENTR-601-45 and suggest potential for further study in patients with DMD amenable to exon 45 skipping.

1349 Selective Regulation of Gene Expression by THO/TREX Complex in Human Neuronal Cells

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THO/TREX complex is a multi-subunit complex conserved from yeast to human. It is known to be required for transcription and nuclear export of some RNAs. Also, THO/TREX complex prevents DNA; RNA hybrid formation (R-loop) to bind nascent RNA for genome stability during transcription. When THO/TREX complex is depleted, R-loop formations are increased, and DNA of R-loop regions is damaged. In this study, we sought to understand how the depletion of THO/TREX complex leads to changes in RNA expression patterns, selective retention of RNAs in the nucleus, and changes in R-loop formation. We observed that knock out of THO/TREX complex using CRISPR led to decreased cell viability. We conducted transcriptome analyses of THO/TREX knock out cells and found that the genes with most reduced expression levels were related to cell cycle. We found that several genes which are highly expressed in the neuronal cells and are retained in the nucleus of knock out cells. Also, we found the regions of R-loop formation are increased in knock out cells. Analyses of clinical data suggested that certain single nucleotide polymorphisms in components of THO complex are associated to severe neurocognitive and growth disorder. However, the molecular mechanism of how THO/TREX complex are related to neuronal disorders is not completely understood. The results in the study suggests that the selective intervention of R-loop formation of select genes that are regulated by THO/TREX may have therapeutic effects for the neurological disease caused by depletion.

1350 Gene Supplementary Therapy in Dent's Disease Mouse Models for Sustained Therapeutic Effects

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Introduction: Type 1 Dent disease is caused by changes in chloride voltage-gated channel 5 (*CLCN5*) gene on chromosome X, which causes the lack or dysfunction of chloride channel ClC-5. Affected subjects show proteinuria and hypercalciuria, and eventually develop end-stage kidney disease. Currently there is no cure for this disease. This work aims to develop a gene therapy for Dent's disease. **Methods:** CRISPR/Cas9-mediated gene mutagenesis was used to develop a *Clcn5* mouse model with 95% of the ClC-5 coding region deleted. Lentiviral vectors were used to deliver human *CLCN5* cDNA into the kidneys of mutant mice by retrograde ureter injection. Urine was collected before and after gene delivery to examine therapeutic effects. **Results:** *1. Evident but short-term therapeutic effects were observed when mutant mice were treated at adulthood.* For the first 3 months, gene therapy greatly ameliorated Dent-like phenotypes, including decreased

urinary calcium and protein excretion. However, the therapeutic effects diminished 4 months after gene therapy, and immune responses to the transgene products most likely caused the loss of gene therapy effects. *2. Long-term therapeutic effects were observed when the mice were treated at new born*. We treated the mutant mice right after birth, when the immune systems of the mice are not developed, and observed evident and long-term (>8 months, the longest time examined) therapeutic effects. 3. *Gene therapy achieved evident therapeutic effects in Dent model mice with dysfunctional CIC-5*. About 1/3 Dent's patients are cause by missense mutations that express dysfunctional CIC-5 protein, which functions as homodimers. Two questions remain unanswered regarding gene supplementary therapy in these subjects: 1) Will the endogenous dysfunctional CIC-5 protein interfere with the function of the exogenous wild type protein and impair the effectiveness of the gene therapy? 2) Will the immune responses be less severe due to the pre-exposure of the immune system to dysfunctional CIC-5 protein? We performed gene therapy in adult mice with dysfunctional CIC-5^{E211A} and observed normalized diuresis after gene therapy. We are currently examining whether long-term therapeutic effects can be achieved in these mice. **Conclusions:** Gene therapy in new born babies could be a strategy to achieve sustained therapeutic effects in subjects affected by Dent's disease. Gene supplementary therapy is effective for Dent's disease caused by missense mutations although CIC-5 functions as homodimers.

1351 What Does the ICHQ5A Revision Mean for Your Cell & Gene Therapy Product?

Danielle DiTirro, Leyla Diaz, Anna Woodward, Armstrong Alison

Contract Testing Services, MilliporeSigma, Rockville, MD

Any Cell & Gene therapy (CGT) manufacturing strategy requires extensive characterization and release testing to assess quality, safety and efficacy of the intended final product. While national regulatory agencies (i.e., FDA, EMA, NMPA, etc.) provide guidance on the type and degree of testing expected the recommendations may not align. To address this, The International Council of Harmonisation (ICH) is a body of pharmaceutical regulatory experts tasked with harmonizing these quality and safety guidelines to benefit the global development of new pharmaceuticals. The ICH's quality chapter on viral safety testing for biological products (ICHQ5A) has recently been revised and now brings into scope a variety of CGT products. While the revision is still in draft the final revision is expected in 2024 and has significant implications to the quality control and viral safety testing accepted and expected for viral-vector, viral-vector modified and donor-derived products. This presentation provides a description of 1) the CGT products now within scope of the ICHQ5A guidance, 2) where alternative technologies can replace traditional testing strategies, 3) the additional viral clearance studies some vector products are expected to complete, and 4) how these changes will influence the viral safety testing strategies for future CGT products. Case studies will be presented to support the implications and changes expected from the ICHQ5A (R2) guidance.

1352 Development of Master iPSCs with Switchable HLA Genes by Megabase-Scale Genome Engineering

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We recently developed a genome engineering platform for cells having long and complex genomes such as human cells, called Universal Knock-in System or UKiS (*Nature Communications* 13, 4219, 2022). UKiS allows for precise substitution of megabase-scale regions into synthetic genomic sequences of our design in allele-specific and scarless manners in human induced pluripotent stem cells (iPSCs); both alleles of your interest in autosomes can be efficiently modified and any unnecessary sequences such as loxP or markers are not left at the engineered regions. UKiS expands our capability of multi-functionalization of human cell for various industrial uses including therapeutic one. We are currently applying this genome engineering platform for generating new types of iPSCs for future allogeneic cell therapy, which avoids virus- and transposon-based integration of transgenes and knockout of B2M and CIITA genes. In this presentation, we introduces two types of master iPSCs that we created to deepen our understanding of HLA-mediated cell-cell interactions between immune and target cells and to develop allogeneic therapeutic cells. The first master cell (HLA-I free master iPSCs) has ~400 kb deletion of all the six HLA class I gene loci and integration of the UKiS donors at both alleles, whereas the second one (All HLA free master iPSCs) has ~1 Mb deletion of all of the six HLA class I and all the 11 HLA class II gene loci and integration of the UKiS donors at both alleles. The UKiS donor alleles can be used as landing pads which can be efficiently replaced with any gene cassettes of your interest. Here, to demonstrate usefulness of our master iPSCs, we created two different iPSC lines from HLA-I free master iPSCs by replacing both UKiS donor alleles with the following gene fragments: 1) anti-CD19 CAR & the entire gene territory of HLA-E*01:01:01 gene containing the signal sequence of HLA-A or 2) the entire gene territories of HLA-A*24:02:01, HLA-B*07:02:01, HLA-C*07:02:01 and HLA-E*01:01:01. In both cases, ~10 kb-long integration of the entire HLA gene territories including promoters and introns was effectively performed and all the integrated HLA genes were expressed in the endogenous manner because B2M was intact. These master iPSCs are advantageous in investigating the HLA-mediated interaction rules between immune cells and their target cells as well as in developing cell modality for allogeneic cell therapy and regenerative medicine.

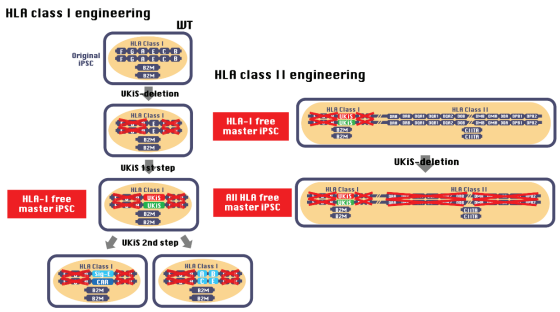

Figure. Schemes of two master iPSC development by Mb-scale genome engineering at HLA locus using Universal Knock-in System (UKiS).

1353 Development of Off-the-Shelf Hematopoietic Stem Cell-Engineered Invariant Natural Killer T Cells for COVID-19 Therapeutic Intervention

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New COVID-19 treatments are desperately needed as case numbers continue to rise and emergent strains threaten vaccine efficacy. Cell therapy has revolutionized cancer treatment and holds much promise in combatting infectious disease, including COVID-19. Invariant natural killer T (iNKT) cells are a rare subset of T cells with potent antiviral and immunoregulatory functions and an excellent safety profile. Current iNKT cell strategies are hindered by the extremely low presence of iNKT cells, and we have developed a platform to overcome this critical limitation. We produced allogeneic HSC-engineered iNKT (AlloHSC-iNKT) cells through TCR engineering of human cord blood CD34+ hematopoietic stem cells (HSCs) and differentiation of these HSCs into iNKT cells in an Ex Vivo HSC- Derived iNKT Cell Culture. We then established in vitro SARS-CoV-2 infection assays to assess AlloHSC-iNKT cell antiviral and anti-hyperinflammation functions. Lastly, using in vitro and in vivo preclinical models, we evaluated AlloHSC-iNKT cell safety and immunogenicity for off-the-shelf application. These results support the development of AlloHSC-iNKT cells as a promis- ing off-the-shelf cell product for treating COVID-19; such a cell product has the potential to target the new emerging SARS-CoV-2 variants as well as the future new emerging viruses.

1354 CRISPR/Cas9 Edited Hematopoietic Stem and Progenitor Cells for Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is a multi-systemic, autosomal recessive neurodegenerative disorder caused by homozygous GAA repeat expansion mutation within intron 1 of the frataxin gene (*FXN*). The mutation reduces FXN expression, a mitochondrial protein required for iron metabolism and respiratory complex assembly. FRDA is characterized by ataxia, areflexia, sensory loss and muscle weakness but cardiomyopathy is the predominant cause of mortality. Symptoms typically begin between 5 to 15 years of age and patients are wheelchair-bound within 10-15 years of onset. Currently, there is no treatment for FRDA. We previously showed complete rescue of neurologic, muscular and cardiac complications of FRDA in YG8R mice with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPC), and rescue was mediated by FXN transfer from engrafted HSPC-derived microglia/macrophages to diseased neurons and cardiac/muscular myocytes. Considering that GAA expansion mutation is seen in 98% of the FRDA patients, is in the intron region, and that overexpression of frataxin is toxic, we developed an ex *vivo* CRISPR/Cas9 gene editing approach for excising the GAA mutation in FRDA patients' $CD34^+$ HSPCs. The present study investigates the *vivo* safety and efficacy of our CRISPR/Cas9 gene editing approach in YG8s$(GAA)_{>800}$ mice model of FRDA that carries human *FXN* (h*FXN*) with >800 GAA repeats. Because the mice carry h*FXN*, we used the same CRISPR/Cas9 components of human $CD34^+$ cells. Lethally irradiated, 2-month-old mice were transplanted with or without *ex vivo* gene edited $Sca1^+$ HSPCs and analyzed 7-month post-transplant. With ~22% of editing efficiency in transplanted $Sca1^+$ HSPCs, we observed ~ 14% gene editing in blood, 4.25% in bone marrow cells, 2.67% in spleen and 4% in thymus. Gene editing in primary organs affected in FRDA such as heart and spinal cord was at ~2% and less than 1% in the brain and skeletal muscle. HSPC-derived cells abundantly increased hFXN protein across the affected tissues, particularly in the spinal cord and heart, despite the limited gene edited level in tissues. Preliminary data suggest decreased pathological cardiac hypertrophy, improved mitochondrial biogenesis, reduced inflammation, and oxidative damage in mice receiving gene-edited cells compared to mock. Altogether, the preliminary data from the *in vivo* studies demonstrate that single infusion of *FXN* gene corrected HSPCs engraft in the bone marrow niche to become a reservoir of healthy cells that can integrate into the injured organs for local and systemic delivery of frataxin protein.

1355 Re-Dosing of Liver-Targeted AAV within and across Clades in Mice: Effects of Neutralizing Antibodies and Vector-Specific Factors

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Recombinant adeno-associated virus (rAAV)-based gene therapy is a promising approach for the treatment of inherited diseases. Since AAV vectors are non-replicating, episomal transgene expression may wane over time, either due to dilution as a result of organ growth in pediatric patients or in response to tissue injury or inflammation. The formation of neutralizing antibodies (nAb) and memory T cells in response to the initial AAV vector treatment may limit the ability to re-dose. Here, we demonstrate that re-dosing among different AAV clades, including Clade F, is possible as evaluated in the the pre-clinical Pah^{enu2} mouse model of phenylketonuria, which has elevated serum phenylalanine (Phe). Two studies are described below. In the first study, the first cohort of mice were dosed intravenously (I.V.) at 5E+12 vg/kg with an AAVHSC15 (Clade F) vector expressing phenylalanine hydroxylase (PAH) and then after 4 weeks, the mice were re-dosed I.V. at 5E+13 vg/kg with AAV5, AAV6 (Clade A), AAV8 (Clade E), AAV9 (Clade F), AAVHSC15 (Clade F), or AAVHSC17 (Clade F) vectors expressing Factor IX (FIX). Blood levels of FIX and Phe were measured throughout the study from Day 3 through Day 98. Mice in the first cohort (initially dosed with 5E+12 vg/kg of AAVHSC15-PAH) showed reduction of serum Phe to normal levels as early as Day 3, and subsequent re-dosing at 4 weeks with 5E+13 vg/kg of AAV5-FIX, AAV6-FIX, or AAV8-FIX (non-Clade F vectors), resulted in successful expression of human FIX in plasma. Re-dosing with a Clade F vector did not lead to normalization of serum Phe levels. In the second cohort, mice were dosed I.V. at 5E+13 vg/kg with AAV5, AAV6, AAV8, AAV9, AAVHSC15, or AAVHSC17 vectors expressing FIX. After 16.6 weeks,

the mice were re-dosed I.V. at 5E+13 vg/kg with AAVHSC15 expressing PAH. Blood levels of FIX and Phe were measured throughout the study from Day 3 through Day 203. Mice in the second cohort (initially dosed with 5E+13 vg/kg of AAV5-FIX, AAV6-FIX, AAV8-FIX, AAV9-FIX, AAVHSC15-FIX or AAVHSC17-FIX) showed expression of human FIX as early as Day 3, and subsequent re-dosing with 5E+13 vg/kg of AAVHSC15-PAH resulted in normalization of serum Phe in mice first dosed with the non-Clade F vectors (AAV5/6/8-FIX) but not with a Clade F vector expressing FIX (AAVHSC15/17-FIX). Interestingly, only one of three mice dosed first with 5E+13 vg/kg AAV8-FIX achieved complete Phe correction. We hypothesized that this variability in the liver transduction efficiency of the second dose could be due to the presence of AAV vector genome (vg) from the first dose. The vg analysis on the terminal liver samples demonstrated that the mouse that exhibited Phe correction post-dose with a Clade F vector had the lowest FIX vg as compared to the mice that did not exhibit Phe correction. In a subsequent study, the interval between the first and second AAV doses was shortened from 16 weeks to 4 weeks. When mice were re-dosed with AAVHSC15-PAH at 4 weeks post-AAV8-FIX dose, every mouse showed successful expression of FIX whereas mice showed little to no reductions in Phe level. These data indicate that the presence of a high level of vg residing in the cells from the first dose in a given timeframe could itself inhibit the transduction of the second dose, which is an additional mechanism beyond the presence of nAb. These data support the potential for successful AAV re-dosing across AAV clades while also highlighting the importance of the initial dose's potency. It is possible that re-dosing with certain clades is dependent not just upon nAb activity, but on the capacity of the liver to be transduced when high levels of AAV vgs are present. Further studies are warranted for translation into human patients.

1356 Toward an *In Vivo* Approach to Knock-Out the HIV Co-Receptor CCR5 in Hematopoietic Stem Cells Using HDAd Vectors Expressing Base Editors

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One of our goals is HIV prophylaxis/therapy by knocking out the HIV co-receptor, CCR5, in hematopoietic stem cells (HSCs) by a simple, cost-efficient, one-day treatment that involves only intravenous injections and could therefore be applicable in resource-poor countries in which AIDS is endemic. Knock-out of CCR5 in HSCs would be passed on to all HSC progeny cells including HIV target cells in the blood and HIV reservoir tissues (gastro-intestinal/genital tracts, brain…). It would therefore block the infection and spread of the virus and lead to a life-long cure/protection. We tested three strategies to knock out CCR5 expression: i) creating a premature stop codon, ii) eliminating the ATG start codon and iii) mutating the splice acceptor sites to skip CCR5 exons. To achieve this, we used base editors (BEs), enzymes that are capable of introducing precise cytidine or adenine substitutions with minimal occurrence of undesired DNA changes at the target site. Here we employed an advanced adenine base editor version (ABE8e) and an early version of cytidine base editor (CBE). ABE8e and CBE are targeted to their desired sites in the genome by a single guide RNA (sgRNA) expressed from a polymerase III U6 promoter. The editing machinery was delivered with helper-dependent adenovirus vectors (HDAd5/35++) that efficiently infect HSCs in vitro and in vivo. Two guide sequences were screened for each of the three strategies. In a CCR5-positive cell line (TZM-bl), transfection with these six all-in-one base editor plasmids generated 13-50% target site editing rates measured by Sanger sequencing. The percentage of CCR5 knockout was 2.93 to 39%. One guide sequence targeting the start codon (referred to as sgSTOP2) and one guide sequence that creates a premature stop codon (referred to as sgR5-1) were demonstrated to mediate higher on-target editing rates as well as CCR5 down-regulation and therefore were selected for downstream experiments. Using miRNA-regulated gene expression and suppression of BE expression with anti-CRISPR inhibitors, we successfully produced two HDAd5/35++ vectors: HDAd-ABE8e-sgSTOP2 and HDAd-CBE-sgR5-1. We then tested the two vectors in TZM-bl cells transduced with increasing vector doses (0-5000 viral particles/cell). Overall, editing rates were higher with the ABE8e vector than with CBE vector, which was most likely due to the higher activity of ABE8e. After infection with HDAd-ABE8e-sgSTOP2 vector at 5000 vp/cell, more than 50% of CCR5 alleles were edited. The percentage of CCR5-negative cells did not differ as much between the two vectors as the editing rate indicating that HDAd-CBE-sgR5-1 blocks CCR5 gene expression more efficiently. Next, we studied whether CCR5 base editing in TZM-bl cells protects them from HIV infection. TZM-bl cells are susceptible to HIV infection (through CD4 and CCR5) and infection can be measured as a function of a reduction in Tat-induced luciferase reporter gene expression. Compared to cells without HDAd transduction, the luciferase activity was 40% and 95% lower in HDAd-ABE8e-sgSTOP2 and HDAd-CBE-sgR5-1 infected cells. This indicates that the HDAd-CBE-sgR5-1 vector is more efficient in blocking HIV infection. The next steps are to further improve this vector by using more active CBE platforms, e.g. TadA-derived CBEs and inserting a $MGMT^{P140K}$ expression cassette that would allow for in vivo expansion of edited HSCs and HSC progeny in animal studies. The improved vectors will then be tested in primary human PBMCs and HSCs that were differentiated into T-cells.

1357 Development of Genome-Modified Generation M (GenM) AAVrh74 Vectors with Improved Transgene Expression in a Mouse Muscle Cell Line and in Primary Human Skeletal Muscle Cells

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We have previously reported that the distal 10-nucleotides (nts) in the AAV2 D-sequence share partial homology to the consensus half-site of the glucocorticoid receptor-binding element (GRE), and that replacement of the distal 10 nts with a full GRE site leads to generation Y (GenY) ssAAVrh74 vectors that mediate up to 6-fold higher transgene expression in primary human skeletal muscle cells compared with those containing the wild-type (WT) D-sequence (*Mol. Ther.*, 30: 237-238, 2022). In the current study, we evaluated if this approach could be extended to include muscle cell-specific transcription factors. The myoblast determination protein 1 (MyoD) and myocyte enhancer factor 2 (MEF2C) have been previously shown

to cooperate in transcription activation during myogenesis (*Mol Cell Biol*., 18: 69-77, 1998). We designed a novel AAV-ITR in which the entire D10-sequence (**Figure 1A**) was replaced with MyoD and MEF2C binding site sequences (**Figure 1B**). Additionally, we replaced part of the stem A-domain to be complementary to the MyoD sequence to ensure that the rerminal resolution site (*trs*) was conserved to allow AAV Rep-mediated resolution, rescue, replication, and packaging. Encapsidation of these genomes containing a muscle cell-specific promoter (MHCK7)-driven hrGFP espression cassette into AAVrh74 capsids led to the development of Generation M (GenM) AAVrh74 vectors. The transduction efficiency of WT and GenM MHCK7-hrGFP AAVrh74 vectors was evaluated in differentiated C2C12 mouse myotubules. As can be seen in **Figure 1C**, the GenM AAVrh74 vectors averaged ~3-fold increased transgene expression compared with that from the WT AAVrh74 vectors (p<0.01). These vectors were further evaluated in differentiated primary human skeletal muscle cells, and shown in **Figure 1D**, mediated ~2-fold increased transgene expression (p<0.01), compared with that from the WT AAVrh74 vectors. These data suggest that genome-modifications involving the AAV D-sequence is a useful strategy to achieve improved transgene expression from ssAAV vectors. Studies are currently underway to optimize the design of the GenM ITR involving an orientation that is speculated to be more conducive of positive transcription factor interaction. Additional studies are also planned involving packaging of GenM genomes into capsid-modified NextGen AAVrh74 vectors leading to the generation of optimized GenM vectors (Opt^{M}) AAVrh74 vectors for high-efficiency transduction as well as augmented transgene expression in murine skeletal muscles following systemic delivery, which has implications in the optimal use of these vectors in the potential gene therapy of muscular dystrophies in humans.

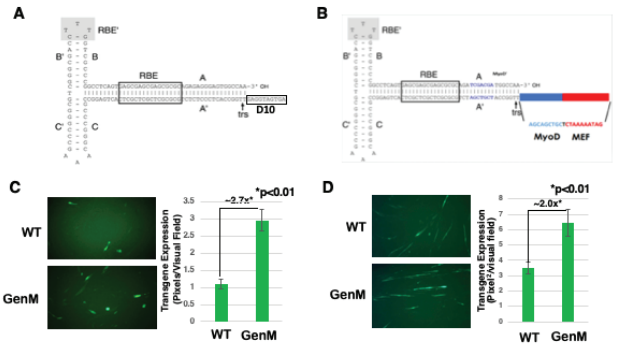

Figure 1: Schematic structures of the WT **(A)** and GenM **(B)** AAV-ITRs. Transduction efficiency of WT and GenM AAVrh74 vectors in differentiated mouse C2C12 **(C)** and primary human skeletal muscle **(D)** cells. Cells were transduced with WT and GenM AAVrh74 vectors at 37°C for 2 hrs, and transgene expression was visualized under a fluorescence microscope following differentiation. Data were quantitated using the NIH ImageJ software.

1358 Gene EXPRESSion Platform: Enabling Early Potency Development and Stability Assessment

Eugenia Fandunyan

Oxford Biomedica Solutions, Bedford, MA

Measuring the potency of your product throughout development is critical for program success. While assays that measure the biological activity of your product are the gold standard for potency assay measurements, these assays typically have long lead times, necessitating an alternative strategy to support early development. We have established a platform gene expression assay to bridge this gap. This poster will walk through the experiments necessary to build a robust gene expression platform assay. The result is a platform assay with a short development timeline for new products (<1 month) that can be used in the early phases of product development. This platform assay can be used to support initial assessments, including candidate screening, scale up comparability, and formulation studies. This poster will also highlight data from a stability indicating assessment that demonstrates that the gene expression platform assay is the most stability indicating assay in our toolbox demonstrating a loss of potency and stability before other analytical methods.

1359 Oncolytic Cell-Based Therapy to Overcome Virus Inactivating Immune Response in Small Cell Lung Cancer Models

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Small cell lung cancer (SCLC) is one of the most detrimental cancers. There are about 30,000 new cases of SCLC patients in the US each year with 80-90% death rates in 2 years. Although many patients experience significant tumor response to their first chemotherapy, these fast-growing cancer cells soon develop chemo-resistance and rebound in a couple of years. Considering these trends, more effective treatments for the SCLC patients are urgently needed including Oncolytic virotherapy (OV). However, OV is found to be restricted by the immune response. Fortunately, with the development of oncolytic virus packaged in stem cells, oncolytic virotherapy now can successfully overcome the barrier of immune response and deliver robust and safe way of tumor killing in patients. In our studies, we loaded clinically safe neural stem cell line (NSCs) with tumor selective oncolytic adenovirus LOAd703 (NSCs. LOAd703) and studied their tumor killing activities in vitro and in vivo. This conditionally replication-competent oncolytic adenovirus was released from NSCs and then selectively replicated within tumor cells and destroyed them in situ via direct lysis. Once lysed, the cells then freed additional viral particles that continued to infect neighboring tumor cells. In addition, this oncolytic virus (OVs) induced tumor cell lysis and consequently exposed the tumor antigens that promoted immune recognition of tumor cells and caused the cascading effect of tumor-killing activities in patients. Through in vitro and in vivo models of humoral immunity-mediated virus inactivation, NSCs. LOAd703 maximized the effect of OV by multiplying the infectious virus particles. They also minimized the blockage of the therapeutic effect of oncolytic viruses (OVs) by the immune barriers and thus protected the OVs from immune mediated inactivation and therefore enhanced the anti-cancer effects.

1360 Engineering AAV Vectors for Sustained Gene Expression in Dividing Cells

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Recombinant adeno-associated viral vectors are powerful tools for *in-vivo* gene delivery. Their non-pathogenic nature, low immunogenicity, and ability to persist episomally without genomic integration make them ideal for gene therapies targeting terminally differentiated cells. However, episomal DNA is lost rapidly in dividing cells, and transgene expression is susceptible to promoter silencing. To overcome these limitations, we designed AAV vectors capable of maintaining themselves episomally in mitotic cells by incorporating scaffold/matrix attachment region (S/MAR) and replication initiation (IR) sequences into an expression cassette. A panel of S/MAR or S/MAR and IR-containing constructs were screened *in-vitro* in Hek293 cells for their ability to sustain gene expression in dividing cells. Eight promising constructs were further evaluated *in-vivo*. CMV-Luciferase reporter constructs were packaged into AAV9 capsid and administrated intravenously to C57/BL6 mice for *in-vivo* assessment. Luciferase reporter gene expression was monitored periodically and terminally by IVIS imaging for 130 days. Three constructs were observed to be superior to controls in maintaining gene expression *in-vivo.* A replicated analysis with three constructs resulted in the identification of single most promising construct. Further analysis was done to map the critical region in the vector for its functionality. A bait-capture deep sequencing study revealed no significant difference in the genomic integration pattern between samples injected with the control vector and the S/MAR-IR vectors. Our modifications to the AAV expression cassette enhanced and sustained expression in dividing cells and *in-vivo,* laying the foundation to expand the utility of AAV vectors

1361 A Cell-Based Potency Method to Assess the Impact of Forced Degradation Methods on AAV Drug Product

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Identification of degradation pathways is a critical aspect of AAV drug product development. A cell-based assay was developed to assess AAV drug product potency and was used to elucidate degradation pathways affecting transduction and protein expression. The vector was subjected to multiple forced degradation processes, high temperature, low pH, high pH and oxidation. Forced degraded vector was tested for capsid titer and viral genome (vg) titer analysis by Gyros and droplet digital PCR, respectively. To determine the transduction efficiency of the force-degraded vector, HEK293 cells were transduced at different MOI's. At 52h post-transduction, the cells were immunostained with a product specific antibody and analyzed by flow cytometry. Gyros assay was performed to quantify the protein expression. All samples were compared to a reference vector lot, non-force degraded. High temperature treatment had no or minimal impact on vg titer and capsid titer, but the vector potency was significantly reduced compared to the reference. Acidic and alkaline pH's reduced the vg titers and capsid titers similarly. The vector potency was significantly reduced for acidic pH treated vector compared to the alkaline pH treated vector. The impact of oxidation on vector was highest when vector was treated with a higher percentage of peroxide, which reduced the vg titer, capsid titer, and vector potency compared to the reference. This assay provides essential insight into the impact of common degradation pathways on AAV drug product potency.

1362 Dendrimer-Based Lipid Nanoparticles for Efficient, Low-Toxicity Gene Editing of Human Hematopoietic Stem and Progenitor Cells

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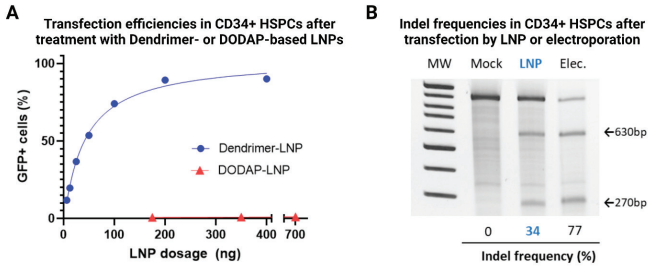
Current gene therapy approaches to the treatment of hematopoietic diseases primarily rely upon *ex vivo* delivery of therapeutic transgenes in human hematopoietic stem and progenitor cells (HSPCs) via integrating retroviral vectors. In contrast, CRISPR/Cas9 technology enables precise editing of cellular genomes, obviating concerns associated with retrovirus-mediated insertional mutagenesis. Additionally, systemic delivery of CRISPR/Cas9-based gene editing cargoes would circumvent the complexity of the *ex vivo* process and the toxicities of preparative regimens necessary for HSPC engraftment after *ex vivo* gene correction. Accordingly, we sought to develop a lipid nanoparticle (LNP)-based strategy for high-efficiency delivery of CRISPR/Cas9 components with minimal cytotoxicity in *ex vivo*-cultured human HSPCs, with the long-term goal of adapting this strategy to targeted delivery of ligand-conjugated LNPs to HSPCs *in vivo*. A recent investigation (*Adv. Mater.* 33, 2006619 [2021]) identified the ionizable amino dendrimer lipid 4A3-SC8 with advantageous properties for gene editing applications. In this study, we characterized LNP formulations based on 4A3-SC8 dendrimers for the ability to deliver various RNA cargos to human HSPCs *in vitro*. As proof-of-concept, LNPs were first encapsulated with GFP-encoding mRNA transcripts at a dendrimer to core lipid molar ratio of 38.5:30:30:1.5 (Dendrimer:DOPE:Cholesterol:DMG-PEG) and validated by physicochemical characterization. Encapsulation using total lipid concentrations of 4-6 mM in the organic phase in combination with low-pH, aqueous phase RNA solutions (nucleic acid concentration 50 ng/uL; pH 3.5) was determined to yield an optimal particle diameter (130-170 nm) and polydispersity index (< 0.2), as well as high encapsulation efficiency (50-90%). To assess the transfection efficiency, LNPs encapsulating GFP mRNA were applied to primary human HSPCs *in vitro,* and GFP expression was quantified by flow cytometry. A dose-response experiment demonstrated highly efficient GFP expression (>80% GFP+ cells) at LNP dosages ≥200 ng per 2.5 x 10e5 cells, with minimal associated cytotoxicity (viabilities 85-99%). In contrast, as characteristically observed with lipid-mediated transfection approaches in primary hematopoietic cells, human HSPCs were refractory to transfection based on LNPs formulated by substituting 4A3-SC8 with the commonly used ionizable cationic lipid DODAP (Fig. A). Moreover, colony forming unit assays of HSPCs transfected

with GFP mRNA-encapsulated 4A3-SC8 LNPs demonstrated preserved colony forming potential. In additional experiments, dendrimer-based LNPs were individually formulated with Cas9 mRNA and single guide RNA (sgRNA) targeting the human AAVS1 locus. Importantly, indel frequencies of up to 34% were achieved in human HSPCs with a single application of Cas9/sgRNA encapsulated LNPs, providing a tractable alternative to electroporation-mediated transfection approaches known to impair HSPC long-term repopulating function *in vivo* (Fig. B). Overall, dendrimer-based LNPs provide a novel low-toxicity and promising platform for human HSPC gene therapy. Future experiments will examine the ability of antibody-conjugated LNPs to target HSPCs *in vivo*.



1363 Peptide Discovery Pipeline Identifies Presented Neoepitope Targets for T Cell Receptor-Gene Therapies

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T cells genetically modified to express a high affinity T cell receptor (TCR) specific for tumor cell-expressed antigens and adoptively transferred into patients have considerable potential for the treatment of cancer. However, substantial uncertainty remains as to which peptide/HLAs should be targeted. To identify protein-derived peptide/HLA pairs that are specific to and continuously expressed on tumor cells, we have developed a pipeline for identifying protein-specific peptides presented across HLA types (ARTEMIS). Briefly, HLA-specific single chain dimers (SCDs), which have a His-Tag instead of the transmembrane domain allowing secretion of the peptide/HLA in the supernatant, are co-transduced with target proteins for downstream mass-spectrometry analysis. We used this versatile platform to identify HLA-specific peptides resulting from point mutations in p53 and alternative splicing events induced by a mutation in SRSF2. Mutations in tumor protein 53 (TP53 or p53) occur in 40-50% of cancer patients. These mutations prevent the function of p53 which controls cell division and cell death and are "driver" mutations on which cancer cells rely for continued proliferation. The top 9 mutations make up approximately 30% of tested cases, making them an ideal target. Previous reports have shown that most common p53 mutation results in presentation of a targetable non-self peptide on HLA-A2. To validate this targeting strategy and expand the eligible patient population, peptide/HLA pairs must be identified for additional mutations across several HLAs. Using the ARTEMIS system, we were able to rapidly screen for mutation-derived peptides bound to 5 different HLAs (HLA-A1, A2, A3, A11, and A24) which cover greater than 90% of phenotype of the US. With SCDs, each individual HLA can be interrogated separately without the complex result deconvolution required by traditional immunoprecipitation methods. Mutations in SRSF2 (Serine and arginine Rich Splicing Factor 2), a protein involved in pre-mRNA splicing, are prevalent across multiple types of high-risk myeloid neoplasms. Experiments with cell lines modified to express a SFSR2-P95H/+ mutation showed mis-regulation of hundreds of splicing events potentially exposing a large pool of unique, tumor-associated peptides that could be targeted by TCR gene therapy. ARTEMIS allows us to directly assess HLA-specific presented neoepitopes resulting from alternate splicing events. We identified thousands of peptides exclusive to mutant P95H/+ K562s cells for HLA-A11 and A24. Next, computational analysis of RNA sequencing data from wild type and P95H/+ cells narrowed the list of peptides to those which resulted from alternate splicing events, against which T cell lines can be developed. This technique can easily be adapted for other cell lines and mutations associated with RNA or protein processing. To validate the identified peptides, we screened for T cell lines against these shared peptide/HLA pairs and assessed their ability to recognize and kill cell lines carrying the p53 or SRSF2 mutations. This work demonstrates a robust pipeline to discover novel HLA/peptide pairs specific to mutated, cancerous cells and thereby extend the reach of engineered TCR-based therapies.

1364 Therapeutic Genome Editing for Tay-Sachs Disease with a Simple CRISPR-Cas9 System

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Introduction: Tay-Sachs disease is a fatal neurodegenerative disorder caused by pathogenic mutations of *HEXA* encoding the α-subunit of Hex-A, an αβ heterodimer that catalyses degradation of GM2 ganglioside in the brain and spinal cord. The most prevalent *HEXA* mutation is a tetranucleotide duplication in exon 11 (1278insTATC), which completely inactivates Hex-A, leading to rapid neurological deterioration from infancy as undegraded GM2 accumulates in neuronal lysosomes. In an engineered cell model, we investigated whether CRISPR-Cas9-induced double-stranded breaks (DSB) within the 1278insTATC duplication could induce productive DNA repair outcomes that restore Hex-A activity. In addition to precise duplication removal via microhomology-mediated end joining (MMEJ) repair following DSB lesions at or near the duplication junction, we posited that therapeutic editing could also occur by non-homologous end joining (NHEJ) insertions/deletions (indels) that re-establish the mRNA open reading frame. Indel-reframed mRNA would yield a Hex-A variant with amino acid alterations yet may still possess physiologically sufficient function. **Methods:** Canonical PAM sequences for natural Cas9 endonuclease species that would permit cutting at or near the 1278insTATC duplication junction were not

present. Thus, we sought to circumvent PAM constraints by using the SpRY nuclease, an engineered variant of *Spy*Cas9 with heavily relaxed PAM requirements. SpRY nuclease activity and concomitant editing were evaluated for 18 guide RNAs (gRNA) targeting all 9 positions on both DNA strands spanning the 1278insTATC duplication. **Findings:** 16 of 18 gRNAs did not yield detectable nuclease activity or generated no therapeutically relevant reframing indels. The gRNA targeting 1nt (nucleotide) downstream of the junction on the antisense strand yielded precise deletion of the duplication at ~19% of all detected alleles alongside a range of non-corrective indels together comprising 67% of all alleles, including 2-nt insertions that theoretically reframe the mRNA found at 22% frequency. Another gRNA, targeting 4nt downstream of the junction on the sense strand, generated a heterogeneous population of non-corrective indels, including a 1-nt deletion at 37% frequency, which is expected to also produce an mRNA-reframed product. Hex-A enzyme activity was fluorometrically assayed using the synthetic Hex-A substrate 4-MUGS to examine how generated editing outcomes ultimately correspond to functional recovery. Perfect deletion of the duplication resulted in full restoration of wildtype Hex-A activity levels. In contrast, three variants with either the 1-nt deletion or 2-nt insertion exhibit little to no recovery of Hex-A function. Western blotting indicates these indels do not lead to high expression of a Hex-A polypeptide, suggesting low tolerance for amino acid perturbations like insertions and substitutions resulting from mRNA-rescuing indels. **Conclusions**: We hypothesise that the lack of therapeutic effectiveness of the reframed mRNA (mutant α-subunit) is due to improper folding leading to reduced stability. Our ongoing mutagenesis work aims to create a larger collection of variants from the 1278insTATC mutation that contain 1-nt deletions putatively restoring mRNA expression. To validate our hypothesis, we will use RT-PCR to confirm the expression of the reframed mRNA and western blot analysis to assess the misfolding issue. The results of this study will provide further insight into the therapeutic potential of CRISPR-Cas9/NHEJ interventions targeting the 1278insTATC mutation.

1365 A Human-Ready Regulated AAV9/mini*MECP2*-miRARE Gene Therapy (TSHA-102) Improves Survival, Weight, and Behavior after Intracerebroventricular (ICV) Dosing in the Neonatal Knockout Rett (RTT) Mouse Model

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Background: Methyl-CpG binding protein 2 (MeCP2) is an X-linked transcription regulator whose loss-of-function and duplication respectively mediate equally severe neurodevelopmental disorders: Rett syndrome (RTT) and MECP2 duplication syndrome. To create an effective gene therapy for RTT while mitigating the risk of overexpression-related side effects, researchers developed a miR-Responsive Autoregulatory Element (miRARE). Insertion of miRARE into the 3'UTR of a mini*MECP2-myc* viral genome was previously shown to improve the therapeutic profile of mini*MECP2-myc* gene therapy. More recently, researchers published an abstract describing the efficacy of TSHA-102, a clinical-candidate version of the AAV9/mini*MECP2*-miRARE vector (lacking the *myc* epitope tag), after intrathecal administration at postnatal day 7 (P7), P14, and P28. To illustrate the full therapeutic potential of TSHA-102, we also evaluated efficacy after neonatal administration. Methods: TSHA-102 was assessed in a pharmacology study in neonatal *Mecp2*$^{-/Y}$ KO mice. This study evaluated the efficacy of TSHA-102 dosing via intracerebroventricular (ICV) route at Postnatal Day 2 (PD2). Efficacy parameters evaluated included body weight (BW), survival, and phenotypic scores (Bird score). Results: ICV administration of TSHA-102 in P2 KO mice at a dose of 8.8 x 10^{10} vg/mouse [Human Equivalent Dose, (HED) 2.86 x 10^{14} vg/participant (Emami et al 2018)] significantly improved survival, BW, and behavior. The median survival for vehicle-treated KO mice was 8.1 weeks. While the longest-lived vehicle-treated KO mouse survived to only 13.3 weeks, ~50% of the treated KO mice survived to 36 weeks of age, the age at which monitoring was ended. TSHA-102 significantly improved BW in KO mice compared to vehicle treated KO mice. Bird score, a composite measure of six different phenotypes, was significantly improved in TSHA-102-treated KO mice. TSHA-102 significantly delayed the average age of onset for severe clasping from approximately 7 to 21 weeks and severely abnormal gait from approximately 8 to 20 weeks. Conclusions: TSHA-102 treatment at 8.8 x 10^{10} vg/mouse extended survival significantly in neonatal *Mecp2*$^{-/Y}$ males, improved weight, improved their overall phenotypic score, and delayed the age of onset for severely abnormal limb clasping and gait.

1366 Scalable Ultra-Purification of Adeno-Associated Viral Vectors - A Novel Standard to Boost Transduction Efficacy and Potency for Cardiac Gene Therapy Development

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Introduction: A high transduction efficacy and potency of AAV-based cardiac gene therapies is key for the clinical translation in which hereditary and acquired cardiac disorders will be targeted. In light of a rather poor cardiac transduction capability of utilized AAVs that led to the premature termination early clinical AAV-based heart failure trials, ultrapure AAV vectors with superior biological potency may be advantageous for the translatability of pre-clinical cardiac gene therapy programs as it allows, e.g., for greater therapeutic efficacy with lower dosages and lower production needs. **Hypothesis:** We hypothesized that our developed affinity chromatography (AC) based purification system will not only increase AAV recovery but also potency of the vectors due to greater purity. **Approach and Results:** Using the same rAAV9 vector input quantity from the upstream HEK-293 production, the AC-based purification enabled an approximately 13-fold greater vector genome copy (vgc) recovery than a density gradient (DG)-

based purification, using iodixanol. Subsequent mass spectrometry analysis demonstrated ultrapure AAV9 vectors as a result of an AC purification (93.24% rAAV9 protein content vs. 6,76% contaminating proteins) whereas corresponding DG preparations resulted in highly contaminated vectors (5.49% rAAV9 protein content vs. 94,51% contaminating proteins), which mirrored results by in-gel silver staining and electron microscopy analysis of both groups. Biological potency of AC- and DG-purified AAV9 vectors towards cardiac transduction efficacy was then determined by systemic tail vein injections of $4{\cdot}10^{11}$, $4{\cdot}10^{12}$ or $4{\cdot}10^{13}$ vgc/kg body weight (BW) of either AC- or DC-purified AAV9-EGFP in C57BL/6 mice (n=8 each group). AC-purified AAVs achieved a significantly higher cardiac transduction efficacy at every dosage level assessed by comparative bulk myocardial viral vector DNA, EGFP RNA and EGFP protein level analysis after 2 weeks by PCR, RT-PCR and immunoblotting. Therapeutic potency was examined using a recently published novel regulatable target system for chronic heart failure, namely the intravenous applicable relaxin (RLN) ligand and RLN regulatable relaxin receptor 1 (RFXP1). Informed by our in vivo cardiac transduction dose-efficacy curve, a dosage of $2{\cdot}10^{12}$ vgcs of either AC- or DG purified AAV9-RXFP1 vectors were systemically injected via the tail vein and the cardiac contractile performance increase was captured after 2 weeks in mice of both groups. Of note, 10 minutes after RLN administration, the rise in LV $+dp/dt_{max.}$ was already significantly greater in the AC- than the DG-vector treated group (AC: 13594+/-1972 vs. DC: 9822+/-801 mmHg/s; n=8 per group, $p<0.01$) and the plateau effect was likewise significantly greater for AC than DG-purified rAAV9-RXFP1. **Conclusion:** Our data clearly promote AC-based AAV purification as a novel standard for cardiovascular basic and translational research. Higher consistency in results, higher therapeutic effects and superior biological potency, e.g., in murine models as demonstrated here, can be expected from higher production yields of ultrapure AAVs already at comparably low clinical dose regimens.

1367 Standardization of Flow Cytometric Measurement on Antigen Expression

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Objectives: Cell-based therapies have emerged as a novel approach to treat cancer and other conditions. Response to antigen-based immunotherapy relies upon the level of antigen expression by tumor cells and decreasing levels of antigen expression can be an early indicator of developing resistance to therapy. For targeted immunotherapy in B-cell malignancies, several studies have highlighted the crucial role of quantifying surface CD19 using flow cytometry in providing guidance for proper selection of therapies. However, the lack of adequate reference materials to control assay variability and the complexity of the cytometer instrumentation, in addition to the existence of numerous cytometer platforms have resulted in a lack of comparable and quantitative CD19 expression analysis across different laboratories and hence, no establishment of clinical cut-off values for CD19 targeted therapies. This study is designed to evaluate CD19 expression as potential reference materials and provide an assessment of their suitability to support the development of CD19 reference standards. **Methods:** CD19 expression measurements were made (1) using QuantiBrite PE calibration with the use of unimolar CD19 PE conjugate and (2) a linearity calibration combined with a single point scale transformation with CD4 as the reference marker. This investigation is an extension of our previous study in which commercially available human peripheral blood mononuclear cells (PBMC) made by three different manufacturers, PBMC-A, PBMC-B, and PBMC-C were evaluated for their suitability as cell reference materials for CD19 expression analysis (https://doi.org/10.1371/journal.pone.0248118). Here, a synthetic CD19 B cell material was tested and compared with PBMC-A. The synthetic CD19 B cell material had three levels of CD19 expression. Variables potentially contributing to the differences in CD19 expression, CD19 PE reagent, lot of PBMC-A and synthetic CD19 cell material, operator, two cytometer platforms, and quantification approaches were considered in the material evaluation. **Results & Conclusions:** Mean values of CD19 ABC (antibodies bound per cell) and associated uncertainty are measured for both PBMC-A and synthetic CD19 cell material. The sources of uncertainty from the variables tested and their relative contributions are identified and analyzed. Full understanding of the uncertainty contributions leads to areas of improvement for production of high-quality and robust reference standards for comparable and quantitative expression measurement using flow cytometry. Antigen quantification assays using ABC values and reference materials evaluated in the study enable the result comparability and establishment of clinical cut-off values, and ultimately provide vital information on patients receiving antigen directed immunotherapy.

1368 A Novel Microenvironment Regulated System CAR-T (MRS.CAR-T) for Immunotherapeutic Treatment of Esophageal Squamous Carcinoma

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The poor efficacy and safety of chimeric antigen receptor T cell therapy due to on-target of tumour effects remain a challenge for application of this therapy in human solid tumors. We designed a tumor microenvironment (TME)-regulated system chimeric antigen receptor T (MRS.CAR-T) which can be selectively activated in the solid TME. B7-H3 was selected as the target antigen for esophageal carcinoma. A conditionally regulated element comprising a human serum albumin

(HSA) binding peptide and a matrix metalloproteases (MMPs) cleavage site was inserted between the 5' terminal signal peptide and single chain fragment variable of the CAR skeleton. HSA binds with an HSA binding peptide effectively, physically preventing systemic activation of MRS. CAR-T cells against B7-H3. MMPs in the TME cleave HSA at the MMP cleavage site and expose the B7-H3 binding site, leading to MRS.CAR-T activation in the TME. The anti-tumor function of MRS.B7-H3.CAR-T cells was better compared to classical B7-H3.CAR-T cells, but reduced IL-2 and IFN-γ expression was noted, reducing the potential for adverse reactions associated with cytokine release. Additionally, MRS.B7-H3. CAR-T produced more effector memory T cells compared to classical CAR-T cells. MRS.CAR-T represents a novel strategy to improve the efficacy and safety of CAR-T therapy in solid tumors.

1369 Development of Cell-Based Assay to Study AAV Vector-Mediated Activation of Innate Immune Responses

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Host immune responses against adeno-associated viral (AAV) vectors pose a safety risk to patients and can reduce therapeutic efficacy of AAV gene therapy. To date, activation of both the innate and the adaptive immune responses against AAV vectors have been well documented in humans during AAV vector-mediated gene therapy. While various strategies have been proposed to reduce the immunogenicity of AAV vectors, due to a lack of predictive in vitro models, these strategies are often tested in animals, which can be time consuming, costly, and often findings do not translate to humans. Thus, development of reliable in vitro assays to assess immune responses against AAV vectors prior to animal studies may help in identifying effective immunomodulatory strategies in a rapid and cost-effective manner. AAV-mediated activation of Toll-like receptor 9 (TLR9) signaling has been shown to contribute to development of both the innate and the adaptive immune responses against AAV vector. However, assessment of AAV-mediated activation of innate immune signaling in vitro has been challenging. Here we screened various human and murine cell lines, along with primary human cells, to assess activation of innate immune signaling pathways by AAV vectors containing single-strand (ss) or self-complementary (sc) genomes. We found that most human and murine cell lines, which are responsive to TLR4, TLR7 or TLR9 ligands or type-I interferons (IFNs), did not respond to AAV vectors. However, primary human monocytes and a THP-1 (human monocyte) cell line were activated to varying degrees by AAV vectors. Interestingly, TLR9 inhibition alone did not completely rescue AAV mediated cellular activation, suggesting TLR9-indepependent mechanisms may also play a role. To further characterize AAV mediated responses in both THP-1 and monocytes, a cytokine array was performed, which identified several novel cytokines upregulated by AAV vectors. Currently, studies are underway to validate identified inflammatory cytokine(s) as a marker of innate immune activation during AAV vector-mediated gene therapy and develop a novel, cost-effective in vitro assay to assess innate immune responses against AAV vectors.

1370 Efficient Messenger RNA Delivery for Universal *Ex Vivo* Modification of Natural Killer Cells for Cancer Immunotherapy

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Natural killer (NK) cells are innate immune effectors that have remarkable potential in eliminating cancerous and virally infected cells via germline-encoded surface receptors. NK cells have gained attention as an alternative to T cells for adoptive cell therapy (ACT), as they can be generated off-the-shelf from allogeneic sources and have shown favorable outcome and lower toxicity *in vivo*. *Ex vivo* engineering of NK cells to express (chimeric) activating receptors or to knock-out inhibitory regulators is a promising therapeutic strategy, as it can enhance their tumor targeting and infiltrating properties. Although viral vectors, including retro-and lentiviruses, have been extensively used to stably deliver genetic material, NK cells are notoriously difficult to transduce. Alternative transfection strategies, such as electroporation, show low cell recovery. Additionally, previous research has shown that electroporated NK cells change their phenotype after treatment. Therefore, advancements in gene delivery strategies are pivotal to achieve efficient NK cell engineering in a safe and regulatory-compliant manner. Our research focuses on the evaluation of mRNA delivery to NK cells via lipid nanoparticles (LNPs). Using microfluidics, we formulated a series of eGFP-mRNA-LNPs altering the cholesterol analogue while tuning the total flow rate and the volume ratio, and we administered them to the NK cell line KHYG-1 to assess both their transfection efficiency and toxicity. All formulations were characterized by size distribution and ζ-potential. Notably, we observed that replacing cholesterol with its natural analogue β-sitosterol resulted in significantly higher transfection efficiency 24 hours after delivery (from 70% to 84%), with a 38-fold increase in the mean fluorescence intensity (MFI) of eGFP expression measured via flow cytometry. Additionally, a decrease in the total flow rate from 11 to 9 mL/min and an increase in the lipid to mRNA volume ratio from 1:1.5 to 1:3 resulted in higher transfection efficiency (93%) and a 4-fold rise in the MFI. Regarding the transfection-related toxicity, treatment with LNPs maintained high cell viability (>99%). When compared to electroporation, transfection via our optimized mRNA-LNP formulation was superior on both KHYG-1 NK and Jurkat T cells. Transfection efficiency of KHYG-1 cells was 92% for LNPs vs 85% for electroporation, with a 35-fold increase in MFI for LNP. Similar results were observed in Jurkat cells, with a transfection efficiency of 95% vs 63% and a 54-fold difference in MFI. Importantly, mRNA delivery with the optimized LNP formulation was also successful in cord-blood derived primary NK cells, GTA002 from Glycostem Therapeutics, resulting in 75% eGFP-positive cells compared to 57% from electroporation. Collectively, these results indicate that, upon optimization, LNPs are an efficient non-toxic mRNA delivery method for *ex vivo* modification of NK cells and show superior transfection potential to electroporation.

1371 Editing the Core Region in HPFH Deletions Alters Fetal and Adult Globin Expression for Treatment of β-Hemoglobinopathies

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Reactivation of fetal hemoglobin (HbF) is the commonly adapted strategy to ameliorate β-hemoglobinopathies. However, the continued production of defective adult hemoglobin (HbA) limits the HbF tetramer production affecting the therapeutic benefits. Here, we evaluated deletional hereditary persistence of fetal hemoglobin (HPFH) mutations and identified an 11 kb sequence, encompassing Putative Repressor Region (PRR) to β-globin Exon-1 (βE1), as the core deletion that ablates HbA and exhibits increased HbF production compared to HPFH or other well-established targets. The PRR-βE1 edited hematopoietic stem and progenitor cells (HSPCs) retained the genome integrity and the engraftment potential to repopulate for long-term hematopoiesis in immunocompromised mice producing HbF^{+ve} cells *in vivo*. Further, PRR-βE1 gene editing is feasible without *ex vivo* HSPC culture. Importantly, the editing induced therapeutically significant levels of HbF to reverse both sickle cell disease (Fig.1 A, B) and β-thalassemia major phenotypes (Fig.1 C, D). These findings imply that the PRR-βE1 gene editing patient HSPCs could lead to improved therapeutic outcomes for β-hemoglobinopathy gene therapy.

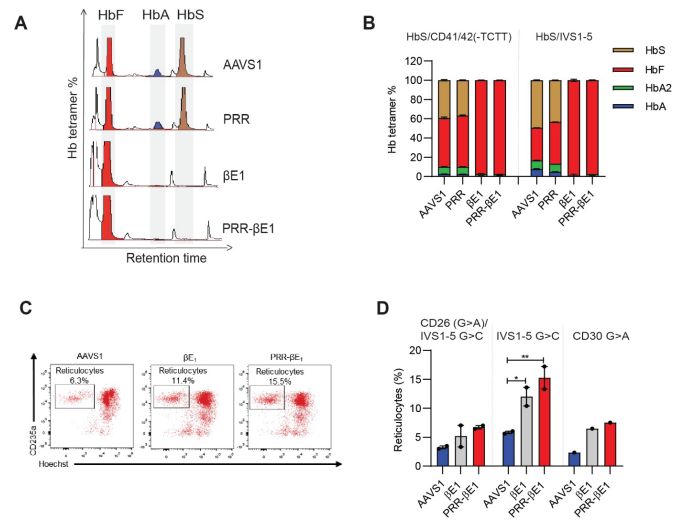

Figure 1 - PRR-βE1 gene editing reverses sickle and β-thalassemia disease phenotype

A. Representative variant HPLC chromatogram showing HbA, HbF and HbS peaks.
B. Proportion of hemoglobin tetramer - the gene edited sickle cell disease patient HSPCs were differentiated into erythroblasts and the hemoglobin tetramers were analyzed by variant HPLC. Donor = 2, n = 2.
C. Representative flow cytometry plots of reticulocytes marked by CD235a+/Hoechst-.
B. Percentage of reticulocytes generated from gene edited β-thalassemia patient HSPCs. Donor = 3, n = 2.

1372 CD20 CAR T-Cells Reversibly Ablate B-Cell Follicles in a Nonhuman Primate Model of HIV Cure

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Introduction: Chimeric antigen receptor (CAR) T cells targeting B cell antigens such as CD19, CD20, and BCMA have generated considerable successes for treatment of B cell malignancies, including lymphomas that are overrepresented in people living with HIV-1 (PLWH). HIV leads to broad immune dysfunction especially in the T cell compartment, which persists even after viral replication is suppressed by antiretroviral therapy (ART). The potency of CAR T-cell therapies for lymphomas and other cancers in PLWH therefore represents a critical gap in knowledge. To address this, we compared the safety and efficacy of CD20 B-cell-specific CAR T cells in our well-established nonhuman primate (NHP) model of ART-suppressed HIV-1 infection. We focused on the impact of $CD20^+$ B cell depletion in tissue-associated B cell follicles (BCFs), key structures where HIV persists on suppressive ART, and which serve as useful markers for CAR T-cell function in solid tissues. The goals of this study were to quantify the impact of suppressed infection on CD20 CAR T-cell function, and conversely to understand how B-cell-targeting CAR T cells may affect persistent HIV infection.
Methods: Using an adapted HIV-like virus (SHIV) to model HIV infection in NHP, we quantified the kinetics, magnitude, and impact of CD20 depletion in 2 groups of 4 pigtail macaques including uninfected and SHIV-infected, ART-suppressed cohorts. NHP CD20 CAR T cells were manufactured *ex vivo* and infused

following low-dose cyclophosphamide conditioning with anti-IL6 prophylaxis. Flow cytometry, PCR-based methods, and immunohistochemistry were used to track CAR T-cell expansion, target cell depletion, and BCF architecture at longitudinal time points *in vivo*. **Results:** CD20 CAR T-cells were well-tolerated and highly functional in both uninfected and SHIV/ART animals with transient B-cell aplasia and reversible BCF ablation observed in both cohorts. SHIV infection was associated with greater sensitivity to lymphodepletion by cyclophosphamide, slower B-cell recovery (uninfected median 46 days, SHIV/ART median 70 days), and lower platelet counts after CAR T-cell therapy. CAR T-cells expanded and trafficked to primary and secondary lymphoid tissues in uninfected and infected animals, but did not appreciably impact SHIV-infected cells in blood and tissues. Across 27 distinct compartments including peripheral blood, no effective reduction of viral reservoirs was observed. CD20 CAR T cells dramatically disrupted BCF architecture in secondary lymphoid tissues; these structures reformed concurrent with rebound in peripheral $CD20^+$ B-cell counts. **Conclusions:** Our findings support the safety and feasibility of CAR T-cell therapies with additional monitoring in PLWH with a B cell malignancy. Although disruption of BCFs in lymph nodes and other secondary lymphoid tissues was insufficient for virus eradication in our HIV model, combinations of B-cell directed CAR T-cells and anti-HIV effector therapies may expose and more efficiently target infected cells in this previously inaccessible reservoir. We also demonstrate that CD20 CAR T-cells reversibly disrupt BCFs, a finding with important implications for CAR T-cell function in solid tissues and for HIV cure approaches. Collectively, these studies validate our promising NHP model of CAR T-cell function, opening numerous avenues to optimize cutting-edge treatments for infectious diseases and cancer.

1374 Synthetic Pathway Activators (SPAs) Increase Engineered T-Cell Potency and Persistence through Tunable STAT Activation

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Clinically effective adoptive T cell therapy for the treatment of solid tumors requires robust T cell expansion, persistence, and potency. The Janus-kinase signal transducer and activator of transcription (JAK-STAT) pathway plays a critical role in governing T cell activation and differentiation, making it a potential axis for programming an effective T cell response against solid tumors. To exploit this potential, we synthetically engineered a library of proteins, termed Synthetic Pathway Activators (SPAs), that can constitutively drive STAT signaling at variable levels without external cytokine input. We have developed several classes of SPAs driving different STAT pathways, including what we term Class I SPAs (SPA.I), which primarily drive the STAT3 pathway. When constitutively expressed in our integrated logic-gated circuit (AND-gated) CAR-T (ICT) cells, SPA.I results in significant enhancements in T-cell potency and expansion evidenced by both in vitro assays and in vivo models. In our repetitive stimulation assays, where T cells are challenged with tumor cells every 2 days for over 2 weeks, SPA.I results in dramatically improved tumor cell clearance. Across various mouse xenograft models, SPA.I-expressing ICTs reach at least 6-fold improved tumor growth inhibition. RNAseq and ATACseq analysis indicate notable changes to gene expression profiles in T cells expressing SPA.I, with maintenance of T cell stem-like phenotypes, and restricted chromatin accessibility at enhancers of various exhaustion marker genes. Importantly, despite significantly increased levels of expansion, ICTs equipped with SPA.I show no signs of cytokine-independent growth. In addition, ICT cells expressing different classes of SPAs show appropriate and rapid contraction following tumor clearance in-vivo. The SPA platform thus allows tuning of T cell biology to engineer T cell therapies with increased anti-tumor potency and cellular persistence.

1375 Characterization of Factors That Influence the Yield and Quality of AAV Produced Using HSV Co-Infection

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Adeno-associated virus (AAV)-mediated gene therapy is a promising clinical approach for the treatment of genetic diseases. Systemic AAV delivery used for gene therapy treatment of muscle diseases such as Duchenne muscular dystrophy requires a high viral dose to deliver the therapeutic transgene to as many muscle fibers as possible. Continual improvements to increase both rAAV quality and yield are thus desired to manufacture sufficient material for clinical use. In this study, several factors were assessed for their impact on rAAV yield and quality in a herpes simplex virus (HSV) production system.Recombinant HSV constructs for the gene of interest (rHSV-GOI) and Rep-Cap (HSV-RC) were used for dual infection of HEK293 cells, cultured in synthetic medium A in a model shake flask system. rAAV yield was measured using GOI ddPCR and quality was estimated through measure of mis-packaged HSV DNA by ddPCR. Additional characterization of relevant proteins from both virus and host cells was also performed to increase understanding of the rAAV production process.Several factors including HEK293 cell density, shaker speed, and the sequence of infection of the two viruses influenced rAAV yield. Multiplicity of infection (MOI) and cell culture medium were identified as the factors with most significant impact on rAAV yield and quality. When the MOI ratio of rHSV-GOI to rHSV-RC was increased 4-fold, both yield and quality were improved. To better understand the MOI-driven rAAV production improvements, ICP0 in HEK293 lysate post-infection was measured as a surrogate for HSV infectivity and demonstrated a clear difference between the two viruses with rHSV-GOI infection of HEK293 cells significantly less efficient than rHSV-RC at the same MOI. Increasing the ratio of rHSV-GOI to rHSV-RC 4-fold achieved similar infection levels of both viruses which correlated to the increased rAAV yield and significantly reduced mispackaged HSV DNA. These data suggest that equal levels of rHSV-GOI and rHSV-RC infection are necessary to facilitate optimal rAAV production and highlight the utility and relevance of characterizing the biology of the HSV infection process. In a study where various culture media were compared, a significantly higher rAAV yield with minimal mispackaging was observed in medium B versus A (used in MOI studies). Improved rAAV

yield and quality in medium B correlated to increased and equivalent levels of ICP0 for both rHSV viruses. Together, these findings highlight the complexity of rAAV manufacture using HSV and identify several factors that significantly influence yield and quality. In particular, the relationship between culture medium and HSV infectivity with its consequent impact on MOI for rAAV production was unexpected and illustrates the value of studies that characterize the biology of HSV infection and rAAV production.

1376 Safety and Preliminary Efficacy of PCRX201, an Intra-Articular Gene Therapy for Knee Osteoarthritis: A Phase 1, Open-Label, Single Ascending Dose Study

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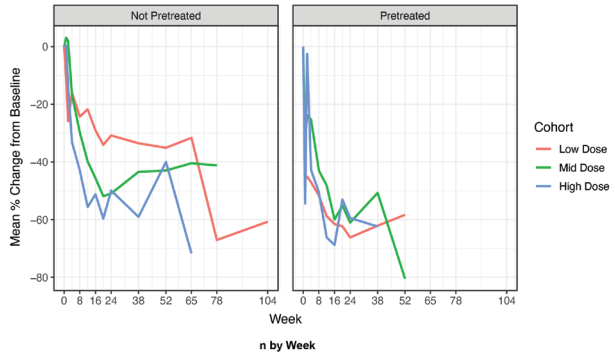
Purpose: Interleukin-1 (IL-1) is believed to be an important driver of inflammation, pain, and disease progression in osteoarthritis (OA). Preclinical data have suggested that gene therapy may offer long-term expression of therapy at the disease site. PCRX201 is a helper-dependent adenovirus that expresses the IL-1 receptor antagonist gene and is designed to be activated by a nuclear factor κB-responsive promoter during inflammation. Herein, we report preliminary phase 1 study results of the safety and efficacy of PCRX201 in knee OA. **Methods:** This proof-of-concept, open-label, single ascending dose study (NCT04119687) enrolled adults (30-80 years old) with moderate-to-severe knee OA. Additional inclusion criteria included Western Ontario and McMaster Universities Osteoarthritis Index pain score (WOMAC-A) ≥4.0 and ≤9.0, Kellgren-Lawrence grades 2 through 4, and prior failure of ≥2 other treatments for OA. The first cohort of participants received ultrasound-guided intra-articular PCRX201 injection to the knee joint at ascending doses (low, mid, and high). The subsequent cohort received pretreatment with intra-articular methylprednisolone 40 mg right before PCRX201 administration at the same doses. Safety was the primary endpoint, including adverse event (AE) monitoring, repeated index knee assessments, and laboratory evaluations. Efficacy was assessed as the change from baseline in pain (WOMAC-A). The current data represent preliminary observable data with no imputations for missing values. **Results:** Thirty-six participants were enrolled and treated in each cohort (nonpretreated, pretreated) between March 2020 and December 2021. Low-, mid-, and high-dose PCRX201 was administered to 12, 16, and 8 patients, respectively, in the nonpretreated cohort and to 13, 15, and 8 participants, respectively, in the pretreated cohort. Index knee effusions considered related to PCRX201 were dose-dependent (50%, 56%, and 100% by ascending dose in the nonpretreated cohort and 31%, 27%, and 63% by ascending dose in the pretreated cohort). Most index knee AEs were treated with rest, ice, acetaminophen, and synovial fluid aspiration. One patient in the pretreated cohort (mid-dose) experienced severe pain during PCRX201 administration, resulting in early injection termination. Pain improvement from baseline was noted for dose groups in each cohort as assessed by WOMAC-A scores (Figure 1). The proportion of participants experiencing a 50% reduction in WOMAC-A from baseline over time is shown in Figure 2. **Conclusions:** PCRX201 was generally well-tolerated, with a decreased severity and incidence of index knee events in the steroid-pretreated cohort versus the nonpretreated cohort. Preliminary efficacy results suggested considerable pain improvement across all doses.

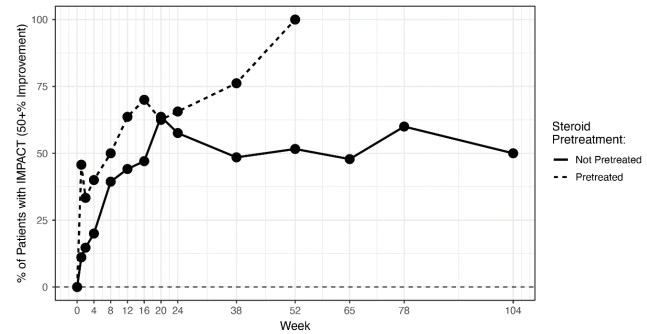
Figure 1. WOMAC-A mean percent change from baseline. Range reflects patients with available data across data points. WOMAC-A, Western Ontario and McMaster Universities Osteoarthritis Index pain score.



n by Week

Treatment	Cohort	0	1	2	4	8	12	16	20	24	38	52	65	78	104
Not Pretreated	Low Dose	12	12	12	12	12	12	12	12	12	12	12	8	4	4
	Mid Dose	16	16	15	15	14	14	14	14	14	14	14	12	6	N/A
	High Dose	8	8	7	8	7	8	8	7	7	7	5	3	N/A	N/A
Pretreated	Low Dose	13	13	13	12	12	12	11	11	11	6	1	N/A	N/A	N/A
	Mid Dose	15	14	15	15	14	13	12	13	13	10	2	N/A	N/A	N/A
	High Dose	8	8	8	8	8	8	7	8	8	5	N/A	N/A	N/A	N/A

Figure 2. WOMAC-A: Percentage of patients with ≥50% decline from baseline. Ranges reflect patients with available data. WOMAC-A, Western Ontario and McMaster Universities Osteoarthritis Index pain score.



n by Week

Treatment	0	1	2	4	8	12	16	20	24	38	52	65	78	104
Not Pretreated	0	4	5	7	13	15	16	21	19	16	16	11	6	2
Pretreated	0	16	12	14	17	21	21	20	21	16	3	N/A	N/A	N/A

1377 A New Benchtop System for Simple and Versatile Introduction of Macromolecules into Human Lymphocytes and CD34+ Cells by Microfluidic Squeezing

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Motivated by the need to address formidable challenges in the study and treatment of diseases, the cell therapy field has recently experienced explosive growth in the availability of novel molecular tools for engineering new cellular functions. A key aspect in the development of these applications is the requirement to efficiently deliver these molecular tools to living cells. The increasing complexity and variety of bio-active molecules that need to be delivered continue to push the limits of traditional methods of intracellular delivery. Indeed, these methods often restrict the range of compatible cargos that can be delivered to primary cell types, particularly with viral transduction and lipid-based transfections. Although electroporation represents a widely used non-viral alternative, significant and unwanted genetic dysregulation that can lead to altered downstream cell biology is often overlooked. In order to address these challenges, we developed the CellPore™ system. This system enables direct cytosolic introduction of molecules to a variety of primary cell types at research scales. The CellPore™ system relies on a mechanism of controlled cell deformation within microfluidic channels under specific flow conditions. This Cell Squeeze® process creates transient disruptions in the plasma membrane that enable direct cargo entry into the cytosol in a gentle fashion. We developed a straightforward workflow to determine optimal cargo delivery conditions to human unactivated T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs). This workflow comprises several steps that include cell isolation, fluorescent cargo preparation for delivery across a range of system pressures, followed by viability and delivery efficiency measurements. Once identified, these optimized parameters were subsequently applied to co-deliver eGFP and mCherry mRNA as well as delivering CRISPR-Cas9 gene editing ribonucleoproteins (RNP) that target the β2 Microglobulin (B2M) gene. Editing of B2M resulted in loss of the major histocompatibility complex (MHC) class I surface molecule. Functional studies of edited cells were also performed in order to determine the impact of the CellPore™ system on overall cell quality. A colony-forming unit (CFU) assay was performed to ascertain the impact of gene editing on HSPC lineage commitment and showed minimal perturbations to their function. Further, we demonstrated that the CellPore™ system did not affect the resting phenotype of unactivated T cells, measured by the absence of both CD69 activation marker expression (0.65 ± 0.1%; mean ± SD; n = 6) and IL-2 cytokine secretion (0.10 ± 0.09 pg/mL; n = 3). This was contrary to results obtained by electroporation that resulted in large increases of both CD69 expression (38.4 ± 4.8%; n = 6) and IL-2 secretion (77.6 ± 23.4 pg/mL; n = 3), independent of T cell receptor (TCR) engagement. Importantly, results also demonstrated the ability of CellPore™-edited unactivated T cells to undergo similar activation and expansion compared to unmanipulated samples. These results underscore the important considerations that need to be made when choosing a suitable intracellular delivery method in order to engineer appropriate cellular function, while limiting unwanted genetic perturbations. The CellPore™ system offers significant advantages over traditional delivery methods in this capacity, in a simple and familiar workflow that can be easily integrated as part of cell therapy research programs.

1378 Brainstem Delivery of a Novel AAV Serotype for Sensorineural Hearing Loss Provides Efficient Gene Transfer Across the Cochlear Spiral of Adult Rats

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Introduction: Noise and chemotherapy-induced hearing loss (NIHL; CIHL) are common types of sensorineural hearing impairment in humans. Cell types that are most vulnerable are hair cells (HCs) and spiral ganglion neurons (SGNs). Gene therapy approaches have focused mostly on restoration or protection of HCs. Such strategies may be effective for hearing loss affecting HCs (e.g. otoferlin mutations), but are unlikely to be effective against NIHL and CIHL. SGNs are the major information pathway from the cochlea to the brain, and their integrity is important to maintain. Key issues limiting the clinical utility of adeno-associated viral (AAV) vector -based gene therapies delivered by direct intracochlear injection are (i) suboptimal delivery of genes across all turns of the cochlea spiral (ii) suboptimal cochlea transduction efficiency in *adult* mammals and (iii) very low levels of SGN transduction. Neurotrophic Factors (NTFs) are of intense interest, and despite relatively modest transduction efficiency with intracochlear delivery (5-10%), up to 14% of the SGN population has been rescued with AAV2-NTF. These studies demonstrate the potential of AAV-based NTF gene therapy and make a compelling case for improving SGN transduction efficiency. We have shown that delivery route can impact AAV-mediated tropism, and that axonal transport can be utilized to deliver genes to brain regions distal from the injection site, the directionality of which is serotype dependent. **Hypothesis:** We hypothesized that targeted delivery of AAV vectors to the brainstem nuclei which innervate the cochlea (cochlea nucleus (CN) and superior olivary complex (SOC)) can be utilized to deliver transgenes to the SGNs in the cochlea via axonal transport. Neurosurgical approaches are considered invasive, however our group has extensive experience in safely targeting AAV-based gene therapies to various subcortical regions in multiple clinical trials utilizing intraoperative MRI guidance and believe the potential improvements in quality of life, present a strong case for development of this approach. **Results:** We show that

AAV-mediated axonal transport can be utilized to transduce SGNs which innervate both IHCs and OHCs with high efficiency across the cochlea spiral following direct intraparenchymal convection enhanced infusion into the SOC and CN of adult rats. An optimized surgical trajectory was developed to avoid damaging brainstem nuclei important for vital life functions. Coclear transduction was observed with the following major AAV serotypes expressing the reporter gene green fluorescent protein (GFP): AAV2, AAV6, AAV9, AAV-Anc80, and a novel AAV serotype developed in-house, AAV-LC.V1 which undergoes bidirectional axonal transport. Transgene delivery was most robust with the AAV-LC.V1 vector, with SGN transduction observed in all cochlear turns (apical, middle and base) at lower titers than those required for the majority of AAV vectors. Transgene expression was limited to neurons, which are not antigen presenting. Importantly, ABR testing revealed infusate delivery to the brainstem of adult rats does not negatively impact hearing. We also show that secreted transgenic GDNF can be taken up by inner HCs, which contain GDNF receptors. **Conclusions:** This study provides proof of concept that targeted AAV delivery to the brainstem can efficiently deliver transgenes to the adult mammalian cochlea via axonal transport.

1379 Development and Validation of a Novel Adeno-Associated Viral Gene Therapy for Mucopolysaccharidosis IIIB (MPSIIIB)

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MPSIIIB is an autosomal recessive lysosomal storage disorder, caused by alpha-N-acetylglucosaminidase (NaGlu) enzymatic deficiency leading to accumulation of Heparan Sulfate Oligosaccharides (HSO) in tissues including the central nervous system (CNS). Patients manifest with early developmental delays followed by severe behavioral abnormalities, progressive neurodegeneration, and death before the age of 20 years. To date, there are no curative therapies for MPSIIIB. We have previously conducted a AAV-2/5 phase I/II intracerebral gene therapy trial that has shown promising results in four MPSIIIB patients with best results being obtained in the youngest patient (18 months-old). However, disease progression in tissues as important as meninges, brain capillary walls, and choroid plexus was presumably not stopped. Therefore, treatment of patients younger than 2 years and the delivery of NAGLU both within and outside the brain was concluded. Here we describe a novel AAV gene therapy using AAVPHP.eB-CAG-NaGlu vector. Following a unique intravenous and intraparenchymal administrations in MPSIIIB mice, high NaGlu activity was measured in the brain, cerebellum, and spinal cord starting 4 weeks post-treatment and persisted after 22 weeks post-treatment. This was accompanied by the normalization of GAG storage levels and improvements in lysosomal pathology and neuroinflammation. AAVPHP.eB-CAG-NaGlu treatment improved motor and cognitive functions. The vector biodistribustion and first assessment of tolerance combining intraparenchymal delivery in the white matter and intravenous delivery was performed in non-human primate. Post-surgery MRI images showed no oedema and perfect tolerance at 1 and 6 weeks post-surgery. A two-fold increase in NaGlu enzyme activity was measured in serum. NaGlu enzymatic activity in the different parts of the brain showed a very significant increase in the white matter, internal capsule, corpus callosum and in the caudate and putamen. Our data indicate that AAVPHP.eB-CAG-NaGlu is a promising treatment option for MPSIIIB patients.

1380 Rationally Engineered Novel AAV Capsids for Intraarticular Gene Delivery

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Arthritis is a group of inflammatory musculoskeletal conditions involving different joints in the body. They usually come with agonizing pain or discomfort that impact daily activities. The current systemic treatments using biological drugs come with various side effects while local treatments often require multiple visits. Due to these drawbacks, intraarticular gene therapy, particularly with adeno-associated virus (AAV) vectors, has been explored to address the most severe problems in local sites. There are three main considerations when AAV vectors are used for intra-articular gene delivery: transduction efficiency in the joint; off target transduction in other tissues, and the existence of AAV neutralizing antibodies (Nabs) in joint fluid. We first screened AAV1-9 vectors encoding firefly luciferase (AAV/luc), 5x109 vg AAV vectors were administered into the knees of C57BL/6 mice. The results from mouse imaging and in vitro luciferase assays showed that AAV 6 had the best knee joint transduction, followed by AAV1, 8, 9, 7, 2, and 5, while AAV3 and AAV4 obtained the lowest transduction. The mice administered with AAV 7, 8, and 9 vectors also displayed luciferase expression in the liver. We also compared AAV transduction under inflammatory and normal conditions using collagen induced arthritis mouse model (CIA), it showed CIA mice exhibited a higher transduction starting from 3mo since priming dose of arthritis induction, till 12mo (our ending point). It was reported that variable region I (VRI) is one of the key determinants for AAV transduction and neutralizing antibody binding, to develop a more effective AAV vector for joint targeted gene delivery, we rationally engineered a set of novel AAV capsids with substitution of VRI between AAV2, AAV6, and AAV8. The results showed the substitution of VRI from AAV6 into either AAV2 or AAV8 increased joint transduction when compared to AAV2 or AAV8 before modification: the mutant AAV2-VRI-6 (created by replacing VRI of AAV2 with that of AAV6) showed a 3-fold higher transduction efficiency than AAV2, and the mutant AAV8-VRI-6 (created by replacing VRI of AAV8 with that of AAV6) had a 2-fold higher transduction efficiency than AAV8. Interestingly, the mutant AAV6-VR1-8 with substitution of VRI from AAV8 into the AAV6 capsid had an 8 -fold lower transduction efficiency than AAV6, while the mutant AAV6-VRI-2 with swapping of VRI from AAV2 into AAV6 induced similar joint transduction to AAV6 with no significant difference. It is worth noting that mice injected with AAV6-VR1-2 displayed lower Nab titers against AAV6 and higher transduction efficiency per copy number than AAV 6. There is only a two amino acids difference between VRIs from AAV2 and AAV6. To further investigate the role of each amino acid in enhancing AAV6 joint transduction, we made two other novel AAV capsids: the mutant AAV6D, with a deletion of the 265 amino acid threonine, and the mutant AAV6M, with a mutation of the 263 amino acid from alanine to glutamine. After direct joint injection, AAV6M experienced a 100-fold decrease in transduction compared to AAV6, while AAV6D induced a 2-fold higher transduction efficiency than AAV6 and AAV6-

VR1-2. Additionally, Nab titers in mice injected with AAV6D were low, with a titer of less than 1:10 in mouse serum. Similar to AAV6, the transgene expression in mice receiving AAV6D vectors was mainly restricted to the injected joints, with less than 0.01 viral particles per cell detected in the liver. Immunohistochemistry staining with rabbit anti-luciferase antibody also demonstrated that AAV6D successfully transduced synoviocytes (29.3±4.5%) and chondrocytes (26.8±4.1%). These results from our study demonstrate that VRI from AAV6 plays an important role in joint transduction efficiency, and that novel AAV mutant AAV6D is able to enhance joint transduction after intra-articular administration, evade AAV Nabs, without crossing the local barrier and entering the blood.

1381 Increasing Feasibility and Safety of Transcutaneous Ultrasound-Mediated Gene Delivery via Intravenous Injection of Plasmids and Microbubbles

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Ultrasound-mediated gene delivery (UMGD) is a promising non-viral method of gene therapy that utilizes therapeutic ultrasound (US) and microbubbles (MBs) to facilitate gene transfer. The current study aims to develop a noninvasive intravenous non-viral gene delivery protocol in combination with transcutaneous US in mouse models to achieve efficient transfection of plasmids in the liver. We embarked our investigation on screening for the optimal formulation of plasmid and MB mixture, the best US settings, and application procedure in a mouse model. A DNA binding study showed that the plasmid DNA can stably bind to our cationic microbubbles (RC2K; 0.011 pg DNA per MB) after 5 minutes incubation time. Thus, a secreted luciferase reporter plasmid was mixed with RC2K MBs for 5 minutes, and subsequently injected into groups of mice via retro-orbital injection with simultaneously transcutaneous application of US to the liver for 60 seconds. Following gene transfer, the transfection efficiency was periodically evaluated by luciferase expression in mouse plasma using the secreted luciferase assay. We found that gene transfection is dose dependent on the luciferase plasmids, reaching a plateau expression level with 50 μg plasmid DNA. Furthermore, a range of RC2K MB percentages (5%-12.5%) can effectively cavitate cells with 10% MBs concentration showing the best transfection efficiency. Additionally, we found that US applied within 30 seconds post-injection of the solution produced higher luciferase expression levels. Furthermore, targeting the liver via scanning on the abdomen was the most effective transducer position that produced the least amount of liver damage to the mice. The US parameters used in our experiments include: a center frequency of 1.1 MHz, a pulse repetition frequency of 14 Hz, and 14 cycles of a 1-second ON and 2-seconds OFF pulse train with a total treatment time of 60 seconds. The peak negative pressure (PNP) of the US was set at 1.5 MPa, and the pulse duration was 150 μs. In our ongoing optimization of the transcutaneous UMGD (tUMGD) technique, we are experimenting with different PNPs and pulse duration values. In most US conditions, we have observed expression levels of up to 5 - 8 x10^4 RLU/mg. Additionally, we are testing the effect of double treatments, and have observed expression levels of over 10^5 RLU/mg protein. In conclusion, tUMGD is a minimally invasive, non-viral, and effective method of gene therapy. By injecting the plasmid DNA/MBs mixture intravenously and applying US to the target organ transcutaneously, this optimized tUMGD technique can be easily translated to treat genetic disorders in clinics.

1382 *In Utero* Pulmonary Gene Editing Persists after Postnatal Lung Injury

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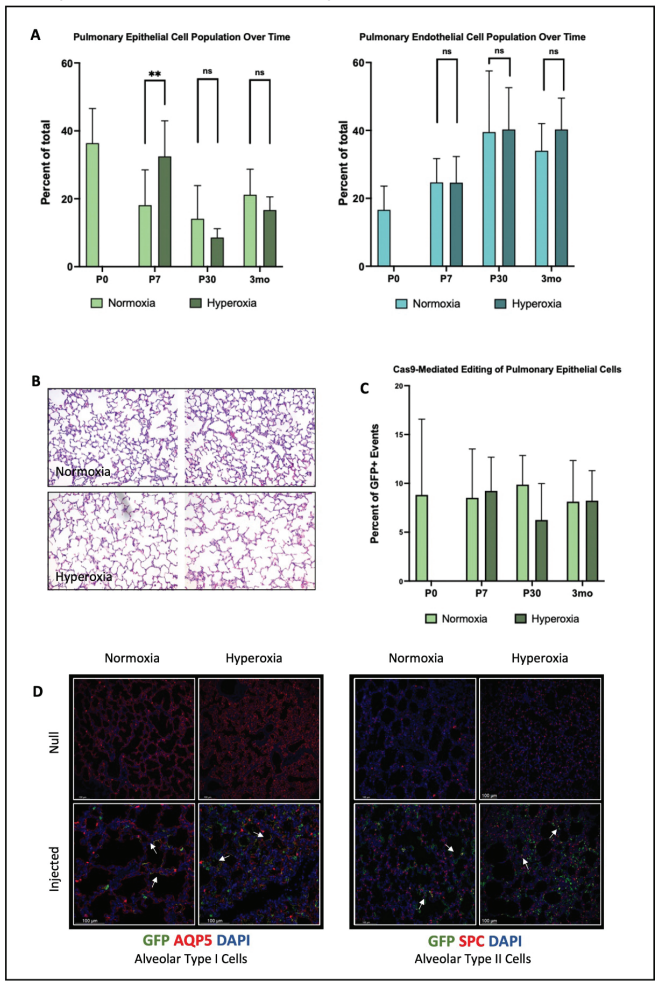
RATIONALE:Monogenic lung diseases are prime targets for *in utero* gene editing to treat and ultimately cure disease prior to the onset of irreversible pathology. Despite novel treatments, patients with congenital pulmonary disorders remain at increased risk of lung injury at birth, whether due to trauma from mechanical ventilation and high oxygen requirements, or increased susceptibility to pulmonary infections. For *in utero* gene therapies to be clinically applicable, it is important to determine whether cells that undergo genetic manipulation retain long-term genotypic stability when subjected to cellular stress. We therefore sought to assess the fate of prenatally edited pulmonary cells in a hyperoxia-induced postnatal lung injury model to determine the persistence of gene editing after postnatal insult. METHODS:The *ROSA^{mTmG}* reporter mouse model constitutively expresses a red fluorescent protein gene that can be excised by Cas9-mediated cleavage, thereby activating expression of green fluorescent protein in all cells that undergo gene editing. Intraamniotic injection of an adenovirus containing SpCas9 and guide-RNA targeting the cleavage sites was performed at gestational day 16. At birth, neonatal mice were placed into either normoxic or hyperoxic (85% oxygen) environments. Hyperoxic treatments lasted seven days after which time all mice were placed in normoxic conditions until the predetermined timepoints. Analysis was carried out at 0, 7, 30 and 90 days. Flow cytometry was performed to quantify pulmonary cell types (epithelial cells (EPCAM+) and endothelial cells (CD31+)) as well as gene editing efficiency based on GFP-positivity. Immunofluorescent microscopy was used to identify whether the expression of alveolar type I (AQP5+) or alveolar type II (SPC+) cells differed after injury and whether gene editing persisted in both cell types. RESULTS: At birth, there were relatively more epithelial cells within the lungs compared to endothelial cells. As the saccular and alveolar stages of lung development progress, there was a relative increase in pulmonary endothelial cells associated with capillary network expansion with a mirrored decrease in epithelial cells as the alveoli expand and develop (Figure 1A). While hyperoxia resulted in alveolar simplification (Figure 1B), there was no difference in relative cellular composition of mouse lungs after injury. Importantly, pulmonary epithelial cell editing remained consistent over time independent of normoxia and hyperoxia exposure (9.8% vs 9.5% at 3 months, respectively) (Figure 1C). Immunofluorescent staining for alveolar type I (AQP5+) and II (SPC+) cells demonstrated GFP positivity in both the normoxia and hyperoxia groups at 90 days, suggesting successful and persistent gene editing of the progenitor type II cells despite injury (Figure 1D, arrows

indicate double positive cells). CONCLUSION:Despite lung injury, prenatal gene editing of pulmonary epithelial cells persists, suggesting neither selection disadvantage nor other detrimental side effects of SpCas9-mediated gene editing exists in the lung. Further, successful editing and subsequent stability of the progenitor cells within the lungs is encouraging as we move towards the development of clinical gene editing approaches to address congenital disease.



1383 Optimization of AAV Transfer Vector for Microglia Specific Transgene Expression

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Microglia, the primary innate immune cells of the central nervous system (CNS), play critical roles in brain development and homeostasis as well as the pathogenesis of many neurological conditions. Transgene delivery to microglia is a promising therapy for CNS injury and disease. Extensive efforts using various types of AAV library screening have identified an increasing number of blood brain barrier (BBB)-crossing serotypes (AAV-B) that can efficiently transduce neurons and astrocytes. Unfortunately, almost all of them failed or had a limited capacity to transduce microglia likely due to the lack of a microglia-specific AAV transfer vector. To help identify ideal AAV serotypes that can efficiently cross BBB and specifically transduce microglia (AAV-BM), we developed a new AAV transfer vector with microglia-specific transgene expression (pDsAAV-HexB-UTR-iCre-WPRE3-miR9T-BGH, 2.418 kb including 2 ITRs). The self-complementary double-stranded AAV transfer vector (DsAAV with packaging capacity around 2.4 kb) was selected to increase the transduction and transgene expression. The minimal/essential promoter of microglia-specific gene HexB (135 bp) was used due to its small size, high specificity and pathological upregulation. The SARS-CoV-2 5'-UTR and the shortest WPRE3 were included to boost the mRNA expression of the transgene. The 4x repeated miRNA-9 target site (miR9T) was added downstream of WPRE3 to illuminate microglia expression (due to miR9 absence) but conceal transgene expression in non-microglia cells that endogenously express miR9, such as neurons, astrocytes, neural stem cells, oligodendrocytes, etc. Finally, the improved Cre (iCre)-mediated LoxP-STOP-LoxP (LSL)-tdTomato (tdT) reporter transgenic animal model (Ai14) was chosen to increase the sensitivity of detecting AAV transduction efficiency because a single copy of iCre delivered by AAV could remove STOP terminator for strong tdT expression. The functions of HexB promoter and miR9T were validated using pDsAAV-HexB-GFP-miR9T vector packaged with AAV2 and AAV-PhP.eB in human microglia and mouse neural stem cells. The packaging efficiency of pDsAAV-HexB-UTR-iCre-WPRE3-miR9T-BGH was assessed by standard 3-plasmid packaging in HEK293T cells with the titer at 1.84e13 GC/ml, comparable to the standard pDsAAV-Cb-GFP. In cultured neural stem cells from Ai14 mice, AAV-iCre-miR9T showed very few positive cells even at 1e6 MOI, while AAV-iCre without miR9T exhibited high transduction efficiency in a dose-dependent manner. In cultured mouse microglia, AAV-iCre-miR9T induced dose-dependent transduction as determined by tdT positivity. Mouse brain stereotactic injection of purified AAV-iCre-miR9T viruses (3.68e10 GC/mouse, 2 µl/site) induced local expression of tdT specifically in microglia. In conclusion, a novel and optimal AAV transfer vector is maximized for its high specificity and sensitivity in transducing microglia both in vitro and in vivo. This vector is useful in screening novel AAV-BM serotypes with BBB-penetration and microglia transduction.

1384 Subretinal Delivery of an AAV Gene Therapy Product Significantly Inhibited the Neovascularization in a Laser-Induced CNV Rhesus Model

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Currently available treatments for neovascular Age-related macular degeneration (wAMD) include Conbercept,Aflibercept, Ranibizumab, Faricimab and Bevacizumab which are antibodies targeting the VEGF axis. These medications are administered regularly and frequently by intravitreal injection, which is an invasive manipulation causing inconvenience to the patients. The purpose of this study is to develop a therapeutic strategy that have a long-lasting effect with reduced administration frequency, potentially even a single administration. We developed a gene therapy medication (KH631) that encodes

anti-VEGF (Conbercept) transgene inside a rAAV8 vector (AAV8-Conbercept). The therapeutic potency of the KH631 was evaluated in a related pharmacodynamic NHP model. Rhesus monkeys were randomly divided into three groups, each animal was administered with KH631 at target dose levels including 3E9, 1E9 and 3E8 vg/eye via subretinal injection respectively. 3 animals were included in each dose group. 28-days post injection animals were subject to laser injuries to their Bruch's membrane, which induce growth of new blood vessels in the subretinal space, mimicking the main characteristics of human wAMD patients. Two animals were used as positive controls which were administrated with 0.5 mg/eye of Conbercept immediately after laser injury. The percentage of grade-4 lesions and the fluorescein leakage (FL) areas of the retina, reflect the neovascular dysfunction severity, and were determined by fluorescein fundus angiography (FFA) at 2- and 4-weeks post damage. At week-2 timepoint post damage, the choroidal neovascularization (CNV) grade-4 lesion inhibition rate was 100%, 91.67% and 73.0% at dosage of 3E9, 1E9 and 3E8 vg/eye groups respectively; in the Conbercept group the inhibition rate was 100%. In the untreated group the number of grade-4 lesion decreased from 36 loci to 28 loci (22.2% reduction). At week-4 timepoint post damage, inhibition was 100%, 94.44% and 90% respectively for the treatment group, and 95.8% for the Conbercept group; the untreated group had 24 loci of grade-4 lesion remaining in total of 38 loci (33.3% reduction). Overall, the FL areas greatly decreased when compared with untreated controls (Figure 1). In conclusion, the data illustrated that a single subretinal injection of KH631 efficiently protected the NHP's retina against CNV related pathological damages. The new gene therapy strategy may potentially have a clinical effect.

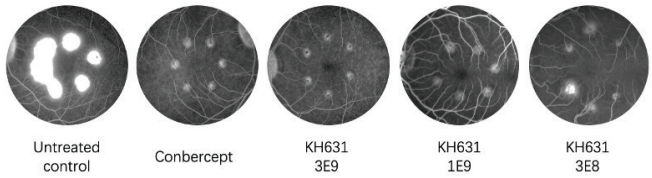

Figure 1. Representative images of fluorescein leakage (FL) test in animals' eyes. KH631 vectors were injected subretinally into rhesus monkeys' both eyes with dosage of 3E8, 1E9 and 3E9 vg/eye respectively; saline was used as the untreated control. Laser modeling was conducted 28 days post injection for all the animals in both eyes; Two animals were laser injured and injected immediately with 0.5 mg/eye of Conbercept as positive controls. FL test was performed at week-4 post modeling, images show the representative images of fluorescein fundus angiography (FFA).

1385 Efficient Gene Correction Strategy for *Recombination Activating Gene 1 (RAG1)* Deficiencies in Human Hematopoietic Stem Cells

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Genome editing based on CRISPR-Cas9 and homology-directed repair (HDR) is a powerful tool for precise *in situ* correction and restoration of the physiological gene regulation, although it is still challenging on hematopoietic stem/progenitor cells (HSPCs). We have developed genome editing strategies to restore the expression and function of the *Recombination Activating Gene 1* (*RAG1*), a key player of the V(D)J recombination process that is tightly regulated during lymphoid differentiation and leads to a spectrum of severe immunological disorders when mutated. A panel of CRISPR/Cas9 nucleases and corrective donors carrying a codon-optimized *RAG1* (co*RAG1*) were screened exploiting a RAG1-deficient cell line (NALM6-*RAG1*.KO) and human mobilized peripheral blood CD34+ HSPCs derived from healthy donors (HD) and RAG1 patients (Pt) carrying biallelic hypomorphic *RAG1* mutations. The first developed genome editing strategy targeted the *RAG1* intron 1 and delivered a co*RAG1* cassette with an upstream splice acceptor sequence to allow transgene expression under the control of the endogenous *RAG1* promoter. Low and variable levels of RAG1 function were achieved in edited NALM6-*RAG1*.KO cells associated with no substantial gene expression modulation in G0/G1 cell cycle phases. Despite the good levels of HDR in edited HD and Pt-derived HSPCs, limited improvement in the lymphoid differentiation after editing of Pt-HSPCs was observed in a 3D *in vitro* model and i*n vivo* in transplanted NOD scid gamma (NSG) mice. Based on these results, we subsequently targeted the *RAG1* exon 2, which is the only coding exon, and developed two different corrective DNA templates to improve the therapeutic potential of our gene correction strategy. Both exon 2 strategies allowed the correction of RAG1 recombination activity and physiological regulation of gene expression in edited NALM6-*RAG1*.KO cells. Importantly, edited Pt-derived HSPCs overcame the T-cell block and differentiated in TCRα/β+CD3+ cells in artificial thymic organoids. *In vivo* experiments in NSG mice transplanted with HD and Pt-derived HSPCs showed engraftment and multilineage differentiation of edited

cells and, importantly, confirmed the efficacy of both exon strategies in overcoming the block of lymphoid differentiation. Moreover, the efficiency of gene correction reached by the exonic editing strategies exceeded the minimal proportion of functional HSPCs that we found to be required to rescue RAG1 immunodeficiency in Rag1$^{-/-}$mice. In conclusion, our findings indicate the feasibility and efficacy of genome editing for the correction of the human *RAG1* locus, strongly supporting its clinical translation for the treatment of RAG1 deficiency, including hypomorphic forms of the disease.

1386 Lipid Nanoparticle Formulations for the Systemic Delivery of CpG-STAT3 siRNA to Myeloid Immune Cell for Cancer Immunotherapy

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We previously demonstrated that eliminating tolerogenic STAT3 signaling from the tumor microenvironment together with Toll-like receptor 9 (TLR9) stimulation results in potent antitumor immune responses. Our proof-of-concept strategy, naked CpG-STAT3siRNA oligonucleotide, proved effective in preclinical studies in mice against human and mouse tumor models, such as leukemia, b cell lymphoma, melanoma, glioma, bladder, and colon cancers. To optimize this strategy for the systemic administration, we utilized MC3-based lipid nanoparticle (LNP) formulations since such delivery systems emerged in recent decade as an attractive tool for gene therapy. After testing LNP compositions varying in N:P ratios and PEG content, we focused on the LNPs with maximal TLR9-mediated immune stimulation as well as target gene silencing. CpG-STAT3siRNA encapsulated in the LNP2 formulation (~100nm in diameter) showed robust type I IFN production (5-10ng/ml of IFNα) in human peripheral blood mononuclear cells (PBMCs) as measured by ELISA. It also resulted in over 50% *STAT3* knockdown in human OCI-Ly3 B cell lymphoma cells as well as in U251 glioma cells at the low oligonucleotide concentration of 100nM in vitro. Importantly, LNP2(CpG-STAT3siRNA) showed ~10× improved potency compared to the naked oligonucleotide against two human lymphoma xenotransplants. Next, we optimized LNP2 formulation using various of anionic helper lipids to maximize targeting of human immune cells. Two of the new LNP2 variants indicated preferential uptake by either human monocytes and dendritic cells (LNP2^{PG}) or by human B cells (LNP2^{CHEMS}). The biodistribution of both LNP2^{PG} and LNP2^{CHEMS} was further verified in biodistribution studies in immunocompetent mice bearing disseminated, syngeneic acute myeloid leukemia (AML). Within 3 h after intravenous injections (1mg/kg), we found significant differences in the cell specific distribution of tested LNPs. As expected, LNP^{PG}(CpG-STAT3siRNA) showed selectivity targeting myeloid cells, including DC subsets, in lymphoid organs such as spleen, lymph node and bone marrow compared to all other formulations. Unlike in human immune cell testing, LNP2^{CHEMS} failed to demonstrate B cell specific targeting in mice. While studies are ongoing, our results so far suggest potential species-specific differences between LNP targeting properties and underscore the need for testing in the context of human immune system in vitro or in humanized mouse models. We believe that the new LNP2^{PG} formulation will provide vehicle for targeting human hematologic malignancies and solid tumors which rely on the tolerogenic myeloid cells for immune evasion.

1387 LT-CRISPR, a Novel Gene-Editing Tool for Precise Editing of the Human Genome

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Genome editing has emerged as a promising strategy to treat various disorders affecting the hematopoietic system. The importance of precise genome editing lies in the fact that it reduces the likelihood of off-target effects, which can cause unintended changes to the genome and lead to harmful side effects. However, therapeutic applications of DNA nucleases suffers from the intrinsically low frequency of homology-directed repair (HDR) compared to Non-homologous end joining (NHEJ) at target site. Presence or absence of different effectors and homologous template at targeted DNA break directs the DNA repair pathway toward HDR or NHEJ. We have demonstrated that by complementing the necessary effectors with Cas9 we can inhibit NHEJ and increase HDR events at targeted site. Using our previously established HDR-CRISPR, we were able to achieve 3-fold increase in precise genome editing as compared to the unmodified Cas9. Here we developed a novel linked-template CRISPR system (LT-CRISPR) to enrich the repair template at DNA double strand break (DSB). We demonstrate that LT-CRISPR is capable of efficiently capturing a single stranded DNA repair template and deliver it directly to the site of the DSB where it promotes DNA repair with 4 fold higher precision for small amino acid change in comparison to unmodified Cas9. Further screening of effectors involved in DNA repair has led to the identification of new fusion partners for the Cas9 that favour the HDR-mediated resolution of a DSB. Ongoing experiments explore the efficacy of LT-CRISPR in fusion with newly identified effectors to correct Fanconi Anemia (FA) specific mutations in-patient-derived hematopoietic stem cells. We believe LT-CRISPR system offers the opportunity to pursue precise genome editing when DNA repair mechanisms are impaired as a consequence of the underlying genetic defect.

1388 AAV Gene Therapy for CSA-Linked Cockayne Syndrome

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Cockayne syndrome (CS) is an autosomal recessive disease caused by mutations in either the CSA (ERCC8) or CSB (ERCC6) gene, leading to defects in transcription-coupled nucleotide excision repair and RNA Pol II mediated transcription. Patients with CS present hypersensitivity to UV light, severe growth failure, dwarfism, short life span and a progressive degeneration of the central nervous system, as well as eye abnormalities (congenital cataracts and retinal degeneration) and hearing loss. The average life expectancy for CS patients is 12 years. Currently there are no therapies for these patients, with the disease being managed symptomatically. CSA is ubiquitously expressed, but its function is not fully understood. It is known to be part of a larger group of proteins with ubiquitin ligase activity thought to regulate both

RNA PolII and CSB. Previous studies have shown that re-expression of normal CSA protein in CSA-associated CS patient cells restores their DNA repair capacity.We designed a panel of self-complementary AAV vectors encoding human CSA under promoters of various strengths. Initial experiments in CRISPR/Cas9 engineered CSA-KO HEK293T cells showed a gradient of protein expression levels assessed by western blot. Accordingly transient transfection of CSA knock-out (KO) HEK293T cells with all AAV constructs restored their survival in a killing assay after treatment with the chemotherapeutic drug Illudin S.Unlike CS patients, CSA-KO mice do not display an overt phenotype and have a normal life span. A severe phenotype is only observed in double CSA/XPA KO mice with very low body weight (< 5 g at weaning) and reach a humane endpoint by 23 days of age. We are testing the therapeutic efficacy of scAAV9-CSA vectors by neonatal ICV injection in CSA/XPA KO mice. Currently several CSA/XPA KO mice treated with the top dose of scAAV9-CSA are ~130 days of age, an increase in lifespan >6-fold (Fig.1), with body weight comparable to that of age matched littermates and no documented adverse events. Importantly the increase in survival is dose dependent. Broad distribution of transduced cells in the brain was documented by immunohistochemical staining for human CSA, and confirmed by western blot analysis of protein expression. Currently we are enrolling more animals in the experiment and testing intermediate doses to identify a minimum effective dose.The success of these AAV proof-of-concept experiments support transition to a clinical trial in CS patients.

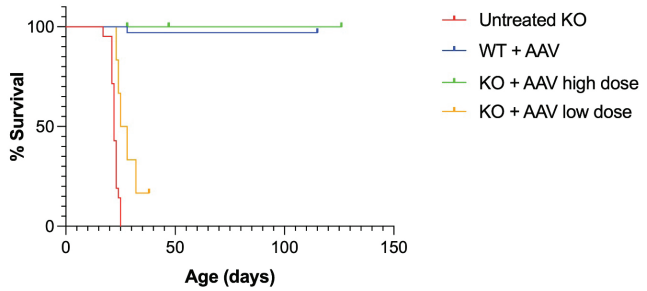

Figure 1. Kaplan-Meier survival plot of CSA/XPA KO mice.

1389 Engineering of the AAVrh.10 Capsid for Cardiac Gene Transfer

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Intravenous administration of AAV serotype rh.10 mediates high level of transduction of the myocardium and is being used to treat hereditary cardiac disorders. However, as with other AAV serotypes, intravenous AAVrh.10 administration also results in high level distribution to the liver which could be a safety concern. The objective of this study was to modify the AAVrh.10 capsid to de-target the liver while retaining effective transduction of the myocardium. Modifications to the AAVrh.10 capsid employed rational engineering. Two variable regions at amino acids 452 and 589 of the capsid VP1 gene were chosen to insert oligopeptides of 5-12 amino acids based on modifications in other AAV serotypes combined with novel molecular modeling. The cardiac and hepatocyte transduction efficiencies of 30 peptide-modified AAVrh.10 capsids expressing the luciferase gene driven by the ubiquitous CAG promoter were first tested *in vitro* in the human cardiomyocyte AC16 and T0539 cell lines and human hepatocyte Huh-7 cell line. Of the initial 30 engineered capsids, 9 had the desired characteristic of de-targeting liver cells while retaining efficient transduction of cardiac cells. Insertion of sequences containing the RGD integrin binding motif in the middle of the inserted peptide in loop VIII helped mediate cardiac-specific transduction. Ten different RGD-containing vectors gave an average expression level 4.0±1.2-fold higher in T0539 cells and 2.8±0.9 higher in AC16 cells compared to all vectors without the RGD motif ($p<0.05$). The optimal *in vitro* engineered capsids were each tested in n=5 C57Bl/6 mice with an intravenous dose $2.5x10^{10}$ genome copies. Two weeks following administration, vector DNA from harvested organs was quantitated. Compared to the wildtype AAVrh.10 capsid, variant M2 (NNPTPSR insertion at position 588) showed a 1.4-fold higher level in heart combined with a 185-fold lower distribution to liver, resulting in a 259-fold higher cardiac to liver specificity. Variant 36.10 (IDGAATK substitution at 452-458 and insertion of ENHTKND at 588) had a 1.3-fold higher level in heart with a 117-fold decrease in liver distribution, resulting in 152-fold increase in heart to liver specificity. In summary, modification of the capsid of AAVrh.10 resulted in effective heart distribution while de-targeting liver. Pending confirmation in larger animals, these vectors may provide an improved organ specificity for cardiac gene therapy.

1390 Dissecting the Effect of Truncated Inverted Terminal Repeats on Productivity, Packaging and Quality of AAV Vectors

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The inherent instability of inverted terminal repeats (ITRs) is one of the major challenges when working with Adeno-associated virus (AAV). ITRs are involved in replication, encapsidation, and integration of AAV genomes. The high GC content and palindromic structure of ITRs facilitates truncations and mutations during plasmid amplification in bacterial cells. It has been shown that even with truncated ITRs, the rAAV genomes can still be replicated efficiently. Deletion of both B-B' and C-C' regions was reported to reduce the rAAV productivity but interestingly increased transgene expression. However, it is more common to observe deletion of either B-B' or C-C', which is known to be less impactful for vector production, as these minor deletions can self-repair during AAV vector genome replication The self-repair is believed to happen when the second ITR is intact, so it can be used as the DNA template during replication. Consistently, our team also observed that B -B' or C-C' region deletion of one of the ITRs showed no significant impact on productivity, packaging efficiency or in vivo potency compared to wild type ITRs. We also observed, using different sequencing methods, that a B-B' deletion in one of the ITRs could be rescued during AAV production. Since self-repairing of the truncated ITR may underestimate the deletion impact, we created a set of vectors with various deletions in the ITR region, at one end and at both ends of the vector genome. We assessed the impact of these deletions in the key regions of the ITR, including the two palindromic loops (B-B' and

C-C'), A region and D region. After producing the vectors using a three-plasmid system transfection of HEK293 cells, the crude harvest lysates were collected and characterized for productivity, full-to-empty capsid ratio, potency, and ITR integrity by sequencing. The result of this work helps to further the understanding of the impact of truncated ITRs on rAAV productivity and qualities during the manufacturing process.

1391 First-in-World Clinical Trial of Genetically Engineered B Cells: Application to the Treatment of Mucopolysaccharidosis Type I

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Due to their capacity for high-level protein production and their natural ability to engraft in bone marrow without need for toxic preconditioning, B cells have the potential to serve as a highly efficacious cellular therapy. Immusoft Corp. has established effective conditions for expansion and genetic engineering of human peripheral blood B cells using the *Sleeping Beauty* (SB) transposon system. Here we present the first genetically engineered B cell product (ISP-001) to achieve US FDA clearance for clinical testing, in this case for the treatment of mucopolysaccharidosis type I (MPS I). MPS I is a rare ($1/10^5$) lysosomal disease caused by absence of α-L-iduronidase (IDUA), leading to systemic accumulation of glycosaminoglycans, organomegaly, skeletal dysplasias, cardiopulmonary obstruction and neurologic impairment. Current treatments by enzyme replacement or by allogeneic hematopoietic stem cell transplant address some but not all of these manifestations. ISP-001 is an autologous B cell product engineered to express human alpha-L-iduronidase (IDUA) for the treatment of MPS I. Manufacturing of ISP-001 starts by collection of the subject's peripheral blood leukocytes by apheresis. The CD19+ population is isolated by immunomagnetic fractionation and expanded in culture for a total of 7 days under defined growth conditions. On day 2 the cells are collected and co-electroporated with an SB transposon construct containing an EEK-regulated human IDUA coding sequence, along with messenger RNA encoding SB100x *Sleeping Beauty* transposase. SB100x transposase expressed from the co-electroporated mRNA then mediates transposition of the SB-EEK-IDUA transposon from the co-electroporated plasmid into the genomes of the expanding B cell population. ISP-001 product release is characterized by flow cytometry, IDUA enzyme level and frequency of IDUA expressing cells, vector copy number and routine safety assessments. Preclinical testing of ISP-001 by xenotransfer into immunodeficient NSG-MPS I mice has demonstrated persistence of IDUA expressing cells and reduced tissue GAG storage for up to six months (the length of the study). The initial clinical trial will be carried out in adults with mild non-neuronopathic disease, testing primarily for safety, with secondary endpoints of assessing biological activity (IDUA, GAG) and initial effects on peripheral manifestations that persist in spite of enzyme replacement. Outcomes from this clinical trial will address the feasibility of this approach for the treatment of MPS I.

1392 A Humanized Mouse Model as a New *In Vivo* Platform to Study and Prevent Pre-Existing Anti-AAV T Cell Responses

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Biotherapies using recombinant adeno-associated viral vectors (rAAV) showed a tremendous success for the treatment of genetic disorders during the last decade. However, rAAV immunotoxicity has been recently reported in patients injected systemically with high doses of viral vectors, leading in some cases to clinical trial holds. Limits imposed by the host immune system emerged when rAAV-based gene transfer protocols were translated from animal models to humans. Indeed, activation of the innate immune system or activation/re-activation of AAV capsid-specific T cells were not reported in preclinical studies despite the fact that, as humans, some large animal models are natural hosts of wild-type AAVs. The absence of relevant preclinical models limits the prediction of success or failure of gene transfer protocols and prevent the development of less immunogenic rAAV vectors or relevant and specific immune suppressive drugs. Development of relevant animal models that mimic immune responses to rAAV vectors observed in patients is crucial for rAAV immunogenicity evaluation and immunomodulation. We developed a humanized rodent model mimicking the cellular immune response described in patients. This model consists in engrafting NSG-HLA-A2+/HHD mice with human PBMCs collected from HLA-A2+ healthy donors with a preexisting anti-AAV8 cellular response and then analyzing the immune response to the capsid after IV injection of a rAAV8 vector. Mice were divided in 3 groups: mice injected with either the vector alone (n=6) or anti-AAV8 IFNγ-secreting cells only (n=3) and, mice injected with both the vector and anti-AAV8 IFNγ secreting cells (n=14, 4 different donors). Our results show an important weight loss only in mice injected with the AAV8 vector and anti-AAV8 IFNγ-secreting human cells, as well as a better engraftment of human CD45+ cells (hCD45+) in the whole blood and the spleen of this same group. These mice also showed an inflammation in the liver and liver infiltrating-cells that are mainly CD8+ T cells in opposition to control groups. A more extensive characterization of these cells is currently ongoing. Since this model appears also relevant to test immunosuppressive strategies, we are currently testing an immunomodulatory strategy using an anti-CD45RC antibody specifically targeting human memory T cell subtypes. In conclusion, this model appears promising to measure the impact of pre-existing anti-AAV T cells on gene transfer efficacy depending on donor's anti-AAV T cell phenotype. It seems also an *in vivo* powerful platform to test immunosuppressive strategies. The final outcome being

to prevent any adverse events related to a defined gene transfer product and define the patient's eligibility to gene therapy clinical trials based on their immune profile.

1393 An Evolved AAV Variant with Enhanced Brain and Spinal Cord Tropism and Translation across Primate Species

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Intravenous delivery of evolved Adeno-Associated Virus (AAV) vectors for gene therapy of the central nervous system (CNS) represents a promising strategy for multiple neurological diseases. The improvements include combining broad transduction of brain and spinal cord tissue together with decreased toxicity risk in comparison with more invasive local administration. Our previously reported TRACER™ capsid discovery platform enables directed evolution and selection of AAVs with improved delivery to various tissues, including CNS, in non-human primates (NHPs). We applied the TRACER™ RNA-driven platform to several peptide display libraries generated by random amino acid substitutions of surface-exposed regions of AAV9. After three rounds of intravenous administration and neuron-specific library RNA selection in cynomolgus macaque, we identified a large collection of capsid variants with improved CNS tropism. In order to evaluate cross-species translatability of these variants, a pool containing the top 145 capsids was tested for CNS tropism in marmosets (*Callithrix jacchus*), African green monkeys (*Chlorocebus sabaeus*) and mouse. Although most variants did not show enhanced brain tropism in those alternative species, the top variant identified in macaques, VCAP-103, recapitulated enhanced CNS transduction in marmosets and AGM, but not in C57Bl/6 or BALB/c mouse. VCAP-103 was further validated as an individual vector in adult cynomolgus macaque using two different therapeutic transgenes, and showed a robust improvement over AAV9, with at least 20-fold higher viral genome biodistribution and transgene RNA expression across the brain and the spinal cord. Immunohistochemical staining demonstrated that this novel capsid allows widespread neuronal transduction in multiple brain regions, including the cortex, putamen, thalamus, and cerebellum, while displaying relatively low transgene expression in liver or dorsal root ganglions (DRGs), despite using a ubiquitous promoter. In summary, this work highlights the possibility of using genetically distant primate species to evaluate the translation potential of evolved AAV capsids, and identifies VCAP-103 as a potential candidate for CNS-targeting applications.

1394 Development of Patient-Derived 3D Tumor Organoids Combined with Immune Cells, as Preclinical Testing Platform for Personalized Cancer Immunotherapy

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Currently available models to validate preclinical efficiency of Immunotherapies lack in mirroring or poorly recapitulate the original patient's tumor. Active immunotherapy, which is dependent on HLA-TCR interaction, cannot be tested in any other system than fully human based *in vitro* systems or humanized mice (complex, expensive, and time-consuming). Plus, only a fraction of patients will gain benefit from immunotherapy treatments and finding responsive patients in advance would be crucial. Therefore, we need to develop more sophisticated systems to address immunotherapies correct mode of actions in humans. Organoids are 3D multicellular structures that retain morphology and function of human tissues and organs. Patient-Derived Tumor Organoids (PDTOs) can be established from primary patient material, cultured long term, and cryopreserved. Successful in recalling the tumor heterogeneity and complexity *in vitro*, PDTOs can provide a robust *ex vivo* system for modelling human cancer, holding great potential as pre-clinical human platform for testing personalized therapies. Our work is focused on combining patient-derived immune cells and PDTOs to develop complex immuno-organoids that retain both patient's tumor unique features and immune system-mediated response. This allows to test tumor cell killing by the patient's own immune system after a personalized immunotherapy treatment. Moreover, after in-depth patient´s tumor characterization, we design a personalized immunotherapy approach to specifically target the patient´s tumor.Oncolytic vaccines have a dual mechanism: direct oncolysis of cancer cells and release of tumor antigens, causing cytotoxic T cell activation against cancer cells. To develop patient-specific oncolytic vaccine-based immunotherapies, our strategy is coating oncolytic vaccines with patient-specific tumor peptides (PeptiCRAd technology). We established PDTOs from Renal Cell Carcinoma and Bladder Cancer, which we characterized by DNA sequencing, immunofluorescence, drug screens. HLA ligandome analysis of the PDTOs allowed the identification of 52 patient-specific tumor peptides. We validated the immunogenicity of these peptides by stimulating healthy donor Peripheral Blood Mononuclear Cells (PBMCs) and analyzing IFN-gamma secretion or CD107a degranulation. We will also use patient-derived PBMCs to unravel the best candidate peptides able to recall a T cell antitumor response. Finally, we will investigate how an oncolytic vaccine decorated with patient-specific tumor peptides (personalized PeptiCRAd) is able to trigger tumor-specific adaptive immune response, measuring specific T cell killing of PDTOs with impedance-based Xcelligence system, LDH release and live cell microscopy. Establishing such a pre-clinical

human model will pave the way for testing patient-specific responses, developing individualized treatments and accelerate their track to the clinic.

1395 The mitoDdCBE System as a Mitochondrial Gene Therapy Approach

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Introduction and Methods The mitoDdCBE system is based on a deaminase whose catalytic domain is capable of deaminating cytosine residues in double-stranded, mitochondrial DNA (mtDNA). To test whether base edited mtDNA can be therapeutic, we took advantage of one of the few existing animal models of heteroplasmic mtDNA mutations, the m.5024T mutant mouse. The m.5024T mtDNA mutation disrupts a Watson & Crick (WC) base pair present in the secondary structure of the mitochondrial tRNA alanine ($tRNA^{Ala}$), reducing its half-life. Because the actual mutation ('T') cannot be corrected, we created a second-site suppressor (at position m.5081C>T) to rescue the phenotype of the m.5024C>T mutation. In this model, an m.5081C>U edited base could restore WC base pairing and improve $tRNA^{Ala}$ stability and function (Fig.1). **Results** To test this hypothesis, we designed *in silico* a pair of mitoDdCBEs to target the m.5081C, electroporated the plasmids carrying the two monomers into a 90% m.5024T mutant line. We then treated the cells with 100ng/ml Ethidium Bromide for 30 days to induce mtDNA depletion and subsequent repopulation. After these treatments, cell colonies were isolated and genotyped. Two clones were selected for further characterization as they have either the lowest (~25%) or highest (~70%) levels of editing achieved while being essentially homoplasmic for the m.5024T. Using a digital PCR (dPCR) approach, we determined the levels of retro-transcribed mitochondrial $tRNA^{Ala}$ and $tRNA^{Asn}$. We found that the $tRNA^{Ala}$/$tRNA^{Asn}$ ratio was inversely proportional to the heteroplasmy levels for m.5024T, and that the edit compensates for the mutation in a dose-dependent way (Fig. 2A). Accordingly, western blotting results showed that the steady state levels of two mitochondrial translation-dependent proteins COX1 and NDUFB8, which are lower in mutant cells, were increased by the base editing (Fig. 2B). Next Generation Sequencing data generated using a novel mim-tRNAseq pipeline confirmed an increase in stability in the edited $tRNA^{Ala}$ and showed that aminoacylation was not affected by the original or edited alterations. **Conclusions** We have successfully edited a cytosine in the mouse mtDNA that acts as a second site suppressor for the pathological m.5024C>T. We are further characterizing the biochemical consequences of the edit and we are currently treating mouse tissues *in vivo* with AAV9 vectors. If successful, we would demonstrate, for the first time, that base edited mtDNA can be therapeutic. **Figure 1. Base editing in the mouse mitochondrial tRNA alanine gene.** The mouse mitochondrial $tRNA^{Ala}$ showing the m.5024C>T mutation (G>A in the $tRNA^{Ala}$). This mutation destabilizes the $tRNA^{Ala}$, probably by disrupting the base pairing at the amino acyl stem. The central image illustrates a putative change in the $tRNA^{Ala}$ secondary structure because of the m.5024 mutation. We have edited the m.5081C to a U, thereby restoring the original WC pairing. **Figure 2. Characterization of the phenotypical consecuences of the m.5081U edit.** (A) Based on the genotype of each cell line, we calculated the probability of having tRNA with a WC pair at the target position. Such value, denoted as 'wild-type mtDNA content', is positively co-related with the steady-state ratio of $tRNA^{Ala}$/$tRNA^{Asn}$. (B) The steady state levels of COX1 and NDUFB8 proteins increased the higher the achieved on-target editing was.

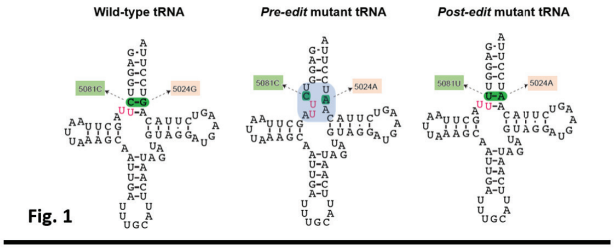

Fig. 1

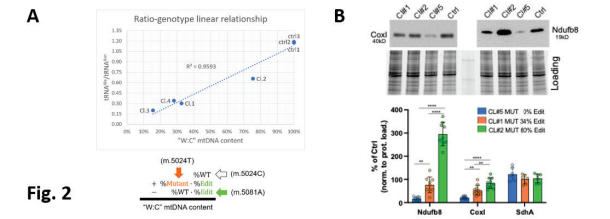

Fig. 2

1396 Autophagy Induction Improves Dual AAV Vector Efficacy in a Mouse Model of Glycogen Storage Disease Type III

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Glycogen storage disease type III (GSDIII) is a rare disease due to mutations in the AGL gene encoding for the glycogen debranching enzyme (GDE). GDE deficiency leads to the accumulation of glycogen mainly in liver, heart, and muscles. With aging the metabolic disorder predominant during childhood is overshadowed by the progressive myopathy and cardiac impairment. To date, there is no curative treatment for GSDIII.The 4.6 kb coding sequence of GDE is a major limitation in the clinical translation of a single AAV-based gene therapy for GSDIII. We previously designed two distinct overlapping AAV vectors with a constitutive or a hepatic promoter, each of them driving the production of the full-length GDE cDNA after recombination, leading to the correction of the muscular and the hepatic phenotype in Agl-/- mice, respectively. We developed an overlapping vector with a liver-muscle tandem promoter able to express GDE (AAV-GDE) in both tissues. Although

efficacy was achieved by a month after injection, the correction was lost overtime. To improve efficacy, we evaluated the combination of AAV gene therapy with an FDA-approved autophagy-inducer drug (Drug Inducing Autophagy, DIA). Treatment with AAV-GDE allowed for complete rescue of the liver phenotype but only partial rescue of the muscle impairment 3 months after injection. However, the combination of DIA and AAV-GDE (AAV-GDE-DIA) increased the correction of glycogen accumulation and muscle strength impairment. To understand the different effects in liver and triceps, we then compared the transcriptomics profiles in these tissues. In triceps, the combined AAV-GDE-DIA treatment induced RNA expression pattern more similar to Agl+/+ mice, while AAV-GDE only partially rescued the RNA expression profile. Interestingly, in triceps, the synergic effect of AAV-GDE and DIA corrects the lysosomal pathway impairment. On the contrary, in the liver, both AAV-GDE and the AAV-GDE-DIA led to RNA expression pattern that differs from both the Agl-/- and Agl+/+ mice, suggesting that restoration of GDE in liver may not results in a complete rescue of the pathology. Together, these results demonstrate that correction of both liver and muscle can be achieved by an overlapping vector expressing GDE with a tandem promoter. Long-term confirmation of the efficacy of the combined treatment is required as well as the validation in other models of the disease to evaluate the robustness of our findings an proceed toward the clinical translation.

1397 CRISPR-Cas9-Mediated Correction of *FANCC* Gene Restores Functions of *FANCC*$^{-/-}$ Hematopoietic Stem Progenitor Cells

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Fanconi Anemia (FA) is an incurable recessive monogenic disease caused by mutations in any member of the FA pathway. Approximately 90% of disease-associated mutations occur in 3 genes (FANCC, FANCA, and FANCG) of the FA core complex. FA group C patients (FANCC mutations) exhibit typical but more severe symptoms compared to other subtypes. Transplantation of hematopoietic stem cells (HSPCs) is a preferred approach to treat bone marrow failure (BFM) in FA patients. However, the lack of matched donors, the enhanced risk of graft-versus-host disease, and the development of secondary tumors are major clinical concerns. These complications can be avoided by using gene therapy to repair/replace mutated genes in autologous FA patient-derived HSPCs (FA-HSPCs). Our overarching goal is to develop a preclinical model for CRISPR/Cas9-based gene therapy to treat BMF in FA group C patients. To this end, we established a universal gene repair system that can rescue all patients by integrating the *Fancc* cDNA into the endogenous *Fancc* promoter of hematopoietic stem cells. Using CRISPR/cas9 as ribonucleoprotein complex and AAV6 as donor template, we efficiently corrected the *Fancc* gene in *Fancc*$^{-/-}$ mouse HSPCs via the homology-directed repair (HDR) pathway. We restored the expression of the *Fancc* gene at the mRNA level. Of note, we showed that typical defective phenotypes of *Fancc*$^{-/-}$ HSPCs (*i.e.,* reduced colony-forming unit and hypersensitivity to mitomycin C (MMC) treatment) were rescued in corrected stem cells. These encouraging data demonstrate that the HDR pathway is active in *Fancc*$^{-/-}$ HPSCs, and we are able to rescue the functions of defective stem cells using CRISPR/Cas9-mediated HDR. We also generated a surrogate model for human FA group C by knocking out (KO) the *FANCC* gene in healthy CD34^{+} cells. *FANCC*-KO CD34^{+} cells exhibit all the typical phenotypes of FA-HSPCs: high level of DNA damage, hypersensitivity to MMC treatment, and reduced colony-forming capacity. This surrogate model is an extremely useful tool to optimize the gene editing system for correcting FANCC mutations in FA-HSPCs. Of note, we also demonstrated the efficient HDR in these cells under the transient presence of DNA-Pk inhibitor (M3814) that suppresses the non-homologous end-joining pathway. Currently, we are validating functions of corrected stem cells in vivo using animal models and exploiting our Spacer-Nick approach (Tran et al, Science Advances, 2022) for a safer gene editing system to correct *FANCC* mutations. In this approach, we use Cas9 Nickase and two off-set PAM-OUT sgRNAs to generate 2 nicks at both strands of targeting sequences. The distance of the two nicks is ranged from 200-350bp minimizing the on-target mutations. We have demosntrated that the Spacer-Nick system sustains high efficient HDR and mitigates all the adverse genetic effects caused by traditional CRISPR/Cas9 system. Additionally, Spacer-Nick induces minimal DNA damage. Thus this approach is suitable for gene correction in *FANCC*$^{-/-}$ cells that are hypersensitive to DNA damage.

1398 Development of AAV Vectors for LGMDR7 Therapy

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Limb girdle muscular dystrophies (LGMD) are progressive, fatal and degenerative genetic disorders. LGMDR7 (formerly known as LGMD2G) is one of the rarest forms of LGMD with autosomal recessive (AR) inheritance affecting both males and females worldwide. LGMDR7 is caused by mutations in the TCAP gene encoding the TCAP (also known as telethonin or T-cap) protein and is characterized by progressive muscle weakness, muscle atrophy, calf hypertrophy, and cardiomyopathy leading to loss of ambulation. There is no cure for patients with LGMDR7. Previously, we developed an rAAV-mediated TCAP construct, the expression of which led to an improvement in the teletoninopathy phenotype and prevented disease progression. Here, we report preliminary data following intramuscular injection of 9-12-week-old humanized TCAPKI-null (hTCAPKI) males and females with several myotrophic rAAV.TCAP vectors. The age matched hTCAPKI and C57Bl/6 mice injected with diluent were used as controls. Treated mice were sacrificed one month post injection to evaluate TCAP protein expression by immunofluorescent (IF) analysis and western blot (WB). Both WB and IF data confirmed a significant restoration of TCAP expression in all vectors. However, hTCAPKI mice treated with muscle-tropic AAV variants showed several folds higher TCAP expression as compared to groups treated with a natural AAV

variant in males and females. These results suggest that myotrophic AAV serotypes could transform the treatment of LGMDR7 patients by significantly reducing vector doses (several logs) and therefore reducing any potential adverse events that can be associated with high AAV doses.

1399 AAV-Mediated Riboswitch-Controlled Delivery of Anti-HER2 Antibody Suppresses HER2-Positive Tumorigenesis

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AAV-mediated antibody expression is a promising therapeutic approach to treating many diseases. However, long-term and excessive amount of therapeutic antibodies from unregulated vector may result in unwanted side effects and a narrow therapeutic window, as well as limiting efficacy of gene therapies. Here, we present the development of regulated anti-HER2 antibody gene, whose expression is controlled by riboswitch via small molecule inducer. Different from previously reported gene regulation systems that involve the use of exogenous protein components, our gene expression platform utilizes a riboswitch which is an RNA element that contains an aptamer as a sensor for small molecule ligand/inducer. In the absence of the small molecule inducer *in vitro*, the antibody gene with a riboswitch cassette does not express antibody protein, whereas in the presence of small molecule inducer, antibody is robustly produced in a dose dependent manner. The riboswitch has been built for activity in mammalian cells and results in activation of gene expression from a very low or undetectable basal level in the absence of the small molecule, to therapeutic levels in a tight dose response to the orally delivered small molecule. When the antibody gene with riboswitch was delivered in AAV to the muscle of mice, orally dosed small molecule induced antibody expression in a tight dose response to the small molecule. Upon withdrawal of daily oral dosing of the small molecule antibody expression subsequently diminished and returned to baseline. The efficacy of small molecule induced anti-HER2 antibody was demonstrated *in vivo* in an HER2+ tumor model. $HER2^+$ tumor growth and tumorigenesis was halted following the daily delivery of the oral small molecule in a dose response to the small molecule, rapidly achieving the same level of tumor blockade as constitutively delivered anti-HER2 antibody. Our data indicate that our synthetic mammalian riboswitch works efficiently *in vivo* and can provide precise control of therapeutic antibody expression by controlling the dose of orally administered small molecule. Expression of the regulated HER2 antibody gene is activated in a dose response to oral small molecule achieving high levels of antibody expression and rapid tumor suppression.

1400 AAV9 Capsid Variants with Improved CNS Transduction Identified by Directed Evolution

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Many monogenic neurological disorders have no approved disease-modifying treatments, owing in part to the poor efficacy of current gene therapy technologies for central nervous system (CNS) tropism. Current methods to counteract poor transduction rely on CNS-directed dosing, yet standard vectors dosed by intrathecal (IT) and intracisternal magna (ICM) routes do not broadly transduce deeper brain structures and at high doses often cause toxicity. The goal of this study is to develop adeno-associated virus 9 (AAV9) capsid variants that more effectively transduce CNS neurons when delivered directly to the CSF, in order to increase vector distribution throughout the brain and reduce vector-associated toxicity. In this study, we describe the application of the CodeEvolver® directed evolution platform to screen for improved AAV9 variants. CodeEvolver® uses high-throughput transfection for capsid production, in vitro screening using human induced pluripotent stem cell (iPSC)-derived CNS cultures, next-generation sequencing, and machine learning combined with other bioinformatics tools to efficiently explore the sequence space and determine the effect of mutations across the entire AAV9 Cap gene. Our screening approach relies on human ex vivo cellular models, as opposed to the rodent or NHP in vivo selections often used for AAV evolution. Unlike in vivo selections, we obtain comprehensive sequence-structure-function data on nearly all variants and avoid the biases of the enrichment and recovery steps of selections. Over three rounds of iterative evolution, we have screened >4000 unique variants and have identified mutations in the capsid proteins that lead to >10-fold improvements in transduction of CNS cells. We demonstrate these improvements in iPSC-derived forebrain neurons, CNS organoid "minibrain" models, and mice. Our results highlight a new approach to capsid engineering and suggest that screening, as an alternative to selection, may be a viable tool for engineering capsids with enhanced properties.

1401 ScFv Mutagenesis to Mitigate CAR-Mediated Tonic Signaling

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Background: In the past decade, chimeric antigen receptor (CAR) T cell therapy has proved to be a valuable tool in our growing armamentarium against cancer, specifically hematologic malignancies. However, efforts to expand this treatment modality into the realm of solid tumors have proved more challenging than initially anticipated. In contrast to hematologic malignancies, solid tumors present numerous tumor-intrinsic obstacles that impede the efficacy of CAR T cell

therapy. In addition, each new target requires the use of a distinct targeting domain, most commonly a single-chain variable fragment (scFv), which poses another set of challenges that are CAR-intrinsic. Although a substantial effort is directed at tackling the tumor-intrinsic obstacles to enable CAR T cell therapy in solid tumors, CAR-intrinsic challenges remain relatively underdiscussed. Arguably, the most important CAR-intrinsic challenge is the ligand-independent basal stimulation resulting from the aggregation of CARs on the cell surface, a phenomenon also known as CAR-mediated tonic signaling. In certain settings, this superfluous signaling has been shown to mimic chronic antigen stimulation and is linked to T cell dysfunction. To date, the most widely adopted strategy to mitigate this issue has been to replace the CD28 costimulatory domain with the 4-1BB domain, which has been shown to result in CAR T cells that are less prone to the deleterious effects of CAR tonic signaling. Crucially, this strategy does not address the problem of CAR aggregation. In this study, we sought to tackle the issue of CAR aggregation through rational mutagenesis of the scFv. **Methods:** An in-silica analysis was used to identify and stabilize potential regions of interest in several publicly available antibodies. ScFvs based on these stabilized antibodies were generated and incorporated into a second-generation CAR construct containing a CD28 costimulatory domain and cloned into a γ-retroviral plasmid by Gibson assembly. Following stimulation, negatively enriched healthy donor T cells were retrovirally transduced with respective CAR constructs and kept in culture for an additional week until immune profiling by flow cytometry and functional assays. Upon profiling, CAR T cells were cocultured with an ovarian carcinoma cell line in an impedance-based real-time cytotoxicity assay. In addition, they were challenged with a variety of recombinant proteins, capable of inducing apoptosis to determine their propensity to enter apoptosis. Throughout the study, an FMC63-derived CD19 targeting CAR and a 14G2a-derived GD2 targeting CAR were used as a negative and positive control, respectively. **Results:** Immune profiling of the scFv-stabilized CAR T cells revealed a less differentiated CAR T cell product while simultaneously demonstrating increased CAR expression on the cell surface, suggesting enhanced stability and diminished CAR-mediated tonic signaling. Additionally, the scFv-stabilized CAR T cells maintained their specificity and demonstrated non-inferiority in their cytotoxic capacity when compared to their non-modified counterparts, as evidenced by a real-time cytotoxicity assay. These findings were further corroborated by their improved resistance to apoptosis when challenged with recombinant proteins. **Conclusion:** Our study suggests that scFv engineering is a viable strategy to stabilize a CAR and curb CAR-mediated tonic signaling. The mutations identified in this work are expected to be broadly applicable to a number of antibody germline families and have the potential to substantially improve the efficacy of CAR T cell therapy by preventing early T cell dysfunction and paving the way for long-term responses.

1402 Point Mutations on Omicron BA.5 Confer Vaccine and Antibody Evasion

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The coronavirus is a quickly evolving virus that has been spreading worldwide, causing 600 million cases and 7 million deaths. As population-wide immunity has developed through vaccinations and infections, SARS-CoV-2 will likely mutate to become more immune evasive, which may cause another coronavirus surge. In this study, we aim to examine the origin of the BA.5 variant (a subvariant of Omicron) and how point mutation frequencies of BA.5 changed from January to October of 2022. We aligned 200 BA.5 nucleotide sequences from the NCBI database and identified their mutation frequency percentages. 83% of all BA.5 variants originated from clade 22B. The point mutations within the spike protein, F486V, L452M, and R493Q, are prevalent in 90% of BA.5 variants. The F486V and L452M point mutations were previously reported to decrease the neutralizing activity of immune sera, which allows BA.5 to escape class 2 and class 3 receptor domain binding. Therefore, the use of monoclonal antibody treatments for Omicron might not be as effective. This study helps immunologists create more effective therapeutics against the BA.5 subvariant.

1403 Making and Breaking the Skeletal Muscle Stem Cell Niche

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Skeletal muscle stem and progenitor cells including those derived from human pluripotent stem cells (hPSCs) offer an avenue towards personalized therapies. Here we demonstrate both stem and progenitor cells readily fuse to form human-mouse myofibers, but skeletal muscle progenitor cells (SMPCs) from hPSCs inefficiently take up position in human-mouse stem cell niches. In contrast, SMPCs expressing the transcription factor PAX7 were 50-fold more likely to associate with a subset of immature human myofibers that resembled fetal niches, 1-2 months after transplantation. We hypothesized lack of SMPC engraftment into the muscle stem cell niche was due to cell competition with endogenous mouse SCs occupying SC niches. Thus, we generated an inducible diphtheria toxin SC ablation mouse model compatible with human engraftment to test this hypothesis. Interestingly, ablation of mouse SCs only increased numbers of immature human myofibers and both PAX7+ SMPCs and SCs now predominantly formed niches with human-only myofibers, instead of residing in chimeric mouse niches, suggesting that cell competition is not the prevailing driver of niche formation. Thus, we profiled SC ablated mice with single nucleus RNA-Seq and identified the absence of a transient myofiber subtype, expressing the fetal actin isoform *Actc1*, that could support Pax7 cells during regeneration. Similarly, the transplanted immature human myofibers strongly expressed ACTC1, and we used spatial RNA-seq to identify key factors driving *de novo* human niche formation, including sarcomere assembly and biosynthesis of fatty acids, improving our ability to support human PAX7 cell repopulation. To demonstrate ACTC1+ myofibers are essential to supporting PAX7 SMPCs, we used CRIPSR/Cas9 to insert a FKBP12-Caspase9 fuse gene in the 3'

end of ACTC1 in hPSCs that were then differentiated into SMPCs and transplanted *in vivo*. Upon Caspase-induced apoptosis, we found a 90% reduction in ACTC1+ myofibers and over a 100-fold decrease in PAX7 cell numbers compared to non-induced engrafted controls. As opposed to other niche systems, where niches are formed by stem cell homing to empty niches, we found that transient regenerating human skeletal muscle is essential to emerging niche formation *in vivo* to support PAX7 cells.

1404 *In Vivo* Expansion of Gene-Targeted Hepatocytes Enables Safe and Durable Transgene Expression

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Targeted transgene insertion is a genome editing approach that could permanently correct a broad range of liver disorders. However, the utility of this strategy is currently limited by imprecise and inefficient repair mechanisms. For example, homology directed repair (HDR) requires cell division, generally limiting targeting of adult liver to ~1% of hepatocytes. The goal of this work is to develop a system for selective expansion of gene-targeted hepatocytes using essential genes. Our system consists of: (1) transient conditioning of the liver by knocking down an essential gene, and (2) delivery of an untargetable version of the essential gene in *cis* with the therapeutic transgene. To test this approach, we used AAV vectors to insert an essential gene - fumarylacetoacetate hydrolase (*FAH*) - in tandem with a fluorescent marker (TdTomato) into a highly expressed locus in the liver (*Apoa1*). Mice were injected with AAV8 vectors encoding CRISPR/Cas9 (AAV-CRISPR) and a Donor template (AAV-Donor) with *FAH* and a TdTomato transgene. Monthly injections of N-acetylgalactosamine (GalNac)-modified siRNA targeting murine *Fah* were used as a conditioning agent to deplete untargeted hepatocytes and drive expansion of correctly repaired cells. Four months later, mice receiving the conditioning siRNA showed a dramatic 5-fold increase in the number of TdTomato positive hepatocytes, versus those receiving the viral vectors alone (~16% vs 3%). Selective expansion could be further improved to >25% of hepatocytes by manipulation of tyrosine catabolism with a high protein diet. To evaluate the translatability and long-term safety of this approach, we inserted a human Factor IX (*FIX*) transgene into the *Apoa1* locus. Following genome editing, the mice were injected monthly with the conditioning siRNA for three months, and then followed for 1 year. Mice subjected to the expansion protocol showed sustained FIX expression over 1 year with a ~5-fold increase as compared to CRISPR/Donor-treated mice. Liver conditioning caused only a transient elevation of ALT at early time points (4 to 8 weeks), which fully resolved over time. Histology revealed no evidence of liver toxicity and full reconstitution of Fah expression. Importantly, the expansion protocol did not increase the risk of tumorigenesis. SMRT-seq analysis at the *Apoa1* locus revealed 20- to 50-fold increase of correct HDR-, 5'-HDR/3'-NHEJ- or partial integration of the Donor cassette in mice undergoing selection with the conditioning siRNA. Preliminary NGS data showed a dramatic reduction in the relative frequency of off-target to on-target editing in the mice undergoing selection. Overall, these data demonstrate a favorable safety profile and durable transgene expression with our selective expansion technology.

1405 Comparison of Orthogonal Approaches Reveal a Consistent Innate Immunogenic Signature of AAV Capsids

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Success of gene therapy for treatment of rare genetic diseases rely heavily on Adeno-Associated Viral (AAV) vectors that provide many attractive features including tissue specific tropism, transduction of quiescent cells and sustained gene expression. However, immune responses to AAV vectors pose a major challenge to successful clinical translation. The capsid as well as encapsulated transgene trigger activation of both innate and adaptive arms of human immune system. While the adaptive immune responses to AAV triggered by B and T cells have been understood to some extent, innate immune activation is very poorly understood. A major challenge in understanding these immune responses lies in the poor translatability of immune responses observed in clinical trials to an ex-vivo setting. To circumvent this challenge and accurately record innate immune responses, we have developed a novel assay that recapitulates innate immune signature in various human donors in response to administration of various AAV serotypes. We have completed a large multi-month donor-capsid matrix screen in human monocytic dendritic cells (moDCs) from healthy donors with this in-house assay to evaluate capsid responses in immune cells from several human donors. We have also validated our in-house assay with a previously established orthogonal in-vitro "MIMIC®" (Modular IMmune In vitro Construct) system. This module, termed the peripheral tissue equivalent (PTE) construct, is a three-dimensional tissue-engineered endothelial cell/collagen matrix culture system that has been previously used to study innate immune activation and cytokine secretion induced by different agonists, vaccines, and biologics. It can screen several human donors in an unbiased automated manner and uses circulating immune cells of individual donors to capture host innate immune response. From both these methods we can sensitively detect a consistent statistically significant increase in 'signature' cytokines IL-1b, TNFa and IL-6 in cells from different donors that were challenged with varied AAV serotypes. Based on the innate immune activation screens, we observed that some donors "responded" to AAV transduction by releasing cytokines whereas other donors were "non-responders" and did not release cytokine upon AAV treatment. Unexpectedly, given that all AAV serotypes expressed the same transgene, we observe that certain AAV capsids are more innate-immunogenic than other capsids at the same dose. These novel findings provide us an early insight into mechanisms responsible for host innate immune responses to gene therapy vectors and will help us develop strategies to mitigate host reactogenicity to AAVs.

1406 Optimization of AAV Vector Design to Reduce Cross-Packaging and ITR Promoter Activity

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RNAi-based gene therapy is a promising approach to treat dominant genetic disorders. Our lab has developed several AAV vector systems to inhibit genes involved in dominant neuromuscular and neurological diseases which have demonstrated efficacy in pre-clinical animal models. To date, we have not seen evidence of toxicity in rodent models and are working to translate several programs, with next steps including IND-enabling safety and biodistribution studies in larger animal models. With this path to translation in mind, we became concerned with a recent publication showing that AAV-delivered RNAi triggers (artificial, therapeutic miRNAs) were safe and effective in mice but showed toxicity in non-human primate (NHP) brain (PMID: 34663988). This toxicity in NHPs was not related to miRNA expression but instead linked to contaminating cross-packaged material and aberrant transcription driven from vector ITRs. Considering these mechanisms, we set out to (1) assess cross-packaging and ITR promoter activity in our vector systems; and (2) design a new vector that could potentially reduce these unwanted artifacts without impacting titers or payload efficacy. To accomplish these goals, we inserted two large, benign DNA fragments to our self-complementary AAV (scAAV) proviral plasmids, located outside the inverted terminal repeat (ITR) regions. Further, we inserted stop codon sequences in all possible reading frames outside the ITRs to prevent aberrant translation of potential transcripts arising from ITR-driven transcription. To assess ITR promoter activity, we generated a construct containing *Renilla* and *Firefly* luciferase expression constructs in the vector backbone lacking promoters, immediately adjacent to the ITRs. Lastly, to prevent any aberrant influence of ITR promoter activity on our intended vector payload, we added transcriptional repressor binding sites between the ITR and U6 promoters used to drive miRNA expression within the vector genome. Vectors and intermediate control constructs were packaged into AAV2 for in vitro transduction experiments. Vector yields were similar to those achieved by our first-generation system. Using our luciferase reporters, we measured significant promoter activity for both the Δtrs ITR and full-length ITRs present in scAAV vectors. Addition of transcriptional repressor binding sites did not reduce expression of either *Renilla* or *Firefly* luciferase. We are currently assessing the percentage and expression of cross-packaged material after in vitro transduction of our new, intermediate, and original vectors in HEK293 cells via Nanopore sequencing and RNA-seq, respectively.

1407 A New Car Against the Intracellular Target MAGEA4 is Capable of Lysing Multiple Myeloma Cells *In Vitro* and *In Vivo*

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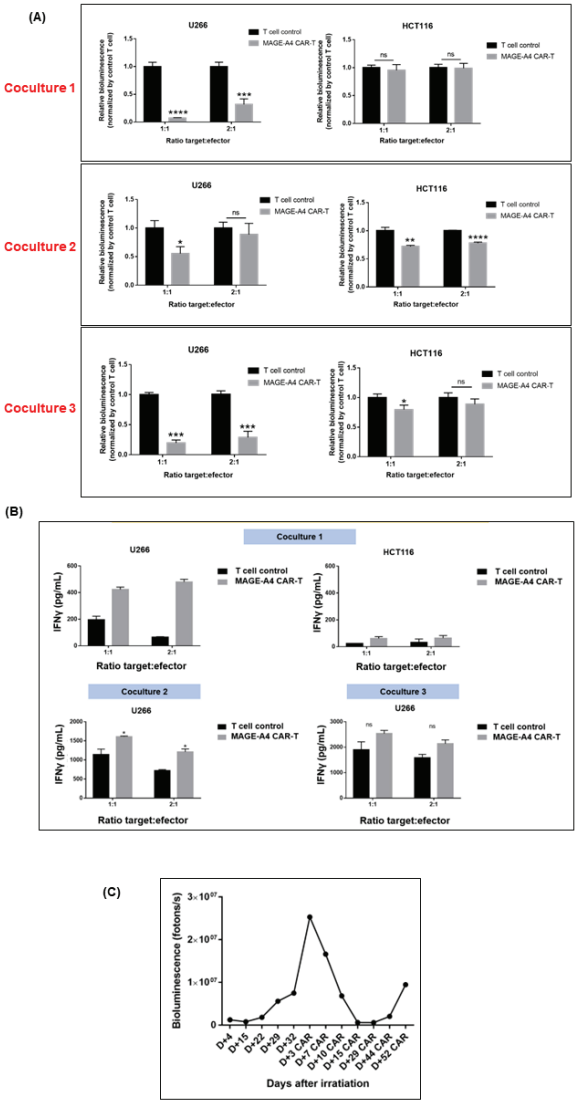
Background: Conventional chimeric antigen receptors (CARs) can recognize only cell-surface antigens in their native conformation. This greatly limits the number of suitable targets since most tumor-associated antigens are intracellular. The T cell receptors (TCR) expressed naturally in T cell usually have low binding affinity, and studies demonstrated that artificially increasing of TCR affinity might lead to unwanted binding to self-antigens. **Aims:** In this work, we report the development of CAR-T cells expressing a novel CAR capable of recognizing the intracellular tumor-associated antigen MAGE-A4 presented by HLA-A*02 molecules, which is the most frequent serotype in the western world. MAGE-A4 is a cancer-testis antigen expressed by a wide myriad of neoplasms, including late-stage multiple myeloma (MM). **Methods:** We generated a novel lentiviral vector coding a CAR harboring a recognition domain derived from an antibody specific to a MAGE-A4:HLA-A*02 complex. Next, we produced lentiviral particles and used them to transduce primary T cells. The resulting MAGE-A4/CAR-T cells were cocultured with luciferase-expressing U266 MM cells (MAGE-A4$^+$) or HCT116 colon carcinoma cells (MAGE-A4$^-$) to assess their antineoplastic potential *in vitro*. We also evaluated IFNγ release during coculture and tested the antitumor potential in a MM xenograft model using NSG mice. **Results:** Transduction efficiencies of new MAGE-A4 CAR vector were high, reaching 57% to 84% in two transductions of different donor samples. These transduced cells displayed normal growth *in vitro* and stable expression of the CAR after 14 days of expansion. The percentage of in vitro tumor cell lysis using CAR-T cells derived from three different donors were 50%, 93% and 45%, respectively. Accordingly, CAR-T cells secreted IFNγ during coculture with U266^{luc} cells, but not with HCT116^{luc} cells, confirming the target-specificity. In an aggressive MM xenograft model, we observed that the MAGE-A4/CAR-T cells induced a strong tumor regression in established high-burden MM grafts, followed by relapse of the diseases after two weeks. **Conclusions:** We demonstrated the generation of a novel lentiviral vector coding a fully functional CAR which was able to redirect the cytotoxic activity of T cells against MAGE-A4+HLA-A*02$^+$ MM cells in vitro and in vivo. These results lay the groundwork for the establishment of a novel advanced cell therapy against MM and other MAGE-A4$^+$ malignancies. Figure 1: Panel with results from co-cultures, ELISA assay and *in vivo* pilot experiment. In A, cocultures were made from three samples from different donors. B, co-culture samples were processed by ELISA to analyze IFN release and, C, viral load was analyzed by relative bioluminescence.



1408 High-Throughput Engineering of Logic Gated-Gene Circuits for Precision CAR Cell Therapies

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Targeted therapies, including chimeric antigen receptor (CAR)-based cell therapies, can cause toxicity when healthy cells also express the targeted protein, making it difficult to make effective and safe therapies against solid tumors. Using CEA-targeted cell therapies in colorectal cancer, for example, has resulted in colitis due to expression of CEA on healthy colon (Parkhurst et al., Mol. Ther., 2011). To address this central challenge in oncology therapeutics, we aimed to develop an inhibitory CAR (iCAR) that recognizes VSIG2, which we demonstrate is expressed selectively on healthy colon epithelial cells. This iCAR blocks CEA CAR-mediated toxicity, thus maximizing the on-target, on-tumor killing of CEA expressing cancer cells. However, CARs are composed of many domains and thus occupy an extremely large combinatorial design space. Current approaches to CAR engineering involve low-throughput batch testing by hand in a time-consuming manner that may be biased by operator inconsistencies. To overcome these limitations, we created a novel automatic liquid handling process, HEPHAESTUS (High-throughput Engineering Platform with Hands-off Automation that Evaluates Smart Therapies with Unbiased Screening), that accepts microwell plates of CAR constructs in DNA form, produces concentrated virus, transduces primary human NK cells, and sets up performance assays using mixed populations of target "healthy" and "cancer" cells. In this assay, NK cells expressing a gene circuit consisting of an anti-CEA activating CAR and an anti-VSIG2 iCAR tended to kill CEA+ "cancer" target cells more than CEA+VSIG2+ "healthy" target cells. We validated the ability of this platform to evaluate and rank the performance of hundreds of gene circuits with different iCARs in parallel, in one self-contained, concurrent, end-to-end process. Manual validation of optimized iCARs confirmed suppression of up to 98% of killing by anti-CEA-CAR-NK cells against CEA+VSIG2+ "healthy" target cells, without any loss of tumor-killing function against CEA+VSIG2- "cancer" target cells. Automated high throughput screening can therefore be used to optimize gene circuits in primary cells to achieve near-digital logic in precision cell therapies.

1409 A Multi-Dimensional Survey of AAV Tropism in Mice

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Adeno-associated viruses (AAVs) have emerged as the leading vector for *in vivo* gene therapy. To expand their utility, it is important to understand the biodistribution and transduction efficiency of different AAV serotypes following delivery. To this end, we undertook a large-scale survey of AAV tropism in mice following systemic administration. AAVs from ten different naturally occurring serotypes of relevance to human gene therapy were examined, including AAV 3b, 4, 5, 6, 7, 8, 9, rh8, rh10, and rh74. Recombinant AAV were packaged to contain a bidirectional transgene cassette for ubiquitous expression of both a ZsGreen fluorescent protein and Cre recombinase. The AAV vectors were tested in"Ai9" reporter mice, which express the tdTomato

fluorescent protein upon Cre-induced recombination. In doing so, we hoped to acquire information about the level of transgene expression (ZsGreen), as well as a highly sensitive means of fate-mapping transduced cells (tdTomato). Multiple mice of both sexes were injected intravenously with 1E12 genome copies of each of the ten serotypes and followed for 4 weeks. A comprehensive panel of 20 tissues (including reproductive organs) from each mouse was prepared for both molecular and histological analysis. To evaluate biodistribution, we quantified vector genome copy number in each organ from every mouse by qPCR. As expected, the majority of AAV serotypes had a strong tropism for the liver. In addition, our data identified several sex differences in transduction efficiency, as well as high tropism of some serotypes in several organs not often considered as targets for AAV transduction. For example, we found that AAV4 strongly transduced lung. Functional tropism analysis by fluorescent imaging has confirmed and extended these results, providing additional information about efficiency of delivery and cell type specific resolution. In addition, a complementary experiment using AAV9 to deliver CRISPR/Cas9 editing reagents demonstrated the adaptability of our approach to genome editing. This work will be publicly available as part of NIH's Somatic Cell Genome Editing (SCGE) initiative, and we anticipate that it will prove to be a valuable resource for researchers aiming to use AAVs to target specific organs and cell types, especially for appropriate selection of capsids in preclinical gene therapy.

1410 Expediting Lentiviral Vector Production with Automated Bioreactors and Design of Experiments

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Background & Aim: Development of new LV bioprocesses is often slow due to the difficulty in establishing optimal values for critical process parameters and the large time requirement to run analytical assays. The ability to make accurate, data-driven product estimations can greatly speed up development timelines. Therefore, the creation of a new LV upstream production process utilized a scalable bioreactor platform with Ambr®15, BioSTAT STR® 50, and Umetrics® Suite data analytics software. The application of design of experiments (DOE) and multivariate data analytics (MVDA) allowed the rapid development and scaling of the LV production process from 15mL to 50L. **Methods, Results & Conclusion:** LVs were produced in HEK293 cells transiently transfected in a 24-way Ambr®15. Infectious titers were calculated by LV transduction on H9 cells measured via flow cytometry. Experiments made use of DOE methodology in MODDE® to design, optimize, and predict bioreactor and process parameters, with results validated in the BioSTAT STR®. The upstream process was developed in two phases. The first phase selected process parameters for the growth and vector production phases of the bioreactor. The second phase further improved, optimized, and characterized the initial process, and then validated the results in a scale-up study. The process was scaled-up from 15mL directly into 50L using the Process Insights software. This project design allowed for rapid deployment of the process for initial GMP facility operations to happen in tandem with powerful process optimization experiments. Data analytics software applying DOE methodology was leveraged to speed up the development timeline by testing and optimizing multiple factors in each experiment. MVDA was utilized to identify process differences between small- and large-scale productions. First phase titers at 50L scale were comparable to those produced at 15mL, and the final optimized process improved upon the initial results. The use of data-based software enabled rapid development of a new LV manufacturing process by reducing the number of experiments required and direct scale-up from 15mL to 50L.

1411 Introduction of Premature Stop Codons within ATXN2 Repeat Induces Therapeutically Relevant Changes in Expression

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Spinocerebellar Ataxia Type 2 (SCA2) is an autosomal dominant disorder impacting up to 1 in every 50,000 individuals. SCA2 is caused by an extended CAG- repeat within exon 1 of the ATXN2 gene that causes ataxia, dementia, and death. Our lab has observed that excising the repeats or disrupting the gene using AAV delivered CRISPR-Cas9 can result in genomic integration of AAV at the cut site. To avoid this possible genotoxic event, we are utilizing base-editing, which can create single-point mutations without the need for a double strand break. We hypothesize that cytosine base-editing can be used to introduce premature stop codons within the repeat region of ATXN2, which will terminate translation and be therapeutically beneficial by reducing mutant ATXN2 protein and RNA levels. Our data shows that base editing can introduce stop-codons within the repeat of ATXN2 *in-vitro,* achieving 47% DNA editing with a corresponding reduction in 33% of RNA and over 80% reduction of ATXN2 protein levels. Based on these exciting preliminary results, we are moving into SCA2 mice to test if this method can alleviate SCA2 disease phenotypes in mouse models. While there are currently no treatments available for SCA2 patients our work shows the therapeutic potential of base-editing to remedy this disease, and future work will focus on patient derived cells available through the Children's Hospital of Pennsylvania.

1413 Optimization of Nuclease Digestion Unit Operation in a Lentiviral Vector Process for Increased Reduction of DNA Impurities

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As the Cell and Gene Therapy (C>) field expands, more and more new innovated therapies and treatments arise. To ensure the safety of these potentially life-changing treatments, one area of focus is around the process-related DNA impurity levels, such as host cell DNA (hcDNA) and plasmid DNA (pDNA), which are typically the concerning process-related impurities for viral vectors. However, due to the complex nature of viral vector production, many factors can inhibit proper reduction of these DNA impurities. Even with the addition of commonly used commercial endonucleases (e.g. Benzonase®),

the desired reduction in the DNA levels might not be sufficient to meet process or regulatory recommendations. Here, we provide the statistical analysis results based on several DoE studies for reduction of hcDNA and pDNA levels in our Lentiviral Vector (LVV) platform process, LentiPeak™. Focusing on several critical parameters including nuclease concentration, supplement concentration, incubation time, and incubation conditions (pH and temperature), this data shows the optimal factors and ranges in the nuclease digestion step. In addition, our data characterizes the impact for each significant parameter to identify the relationship toward DNA impurities reduction for both in-process samples and in the final LVV pool to fully understand the carry through impact of the nuclease digestion step. With the increasing need of viral vectors in the C> field, many upstream processes are evolving to meet production demands with higher cell densities and higher plasmid DNA concentrations. This factor, coupled with industry driven prioritization for patient safety, creates a need for processes, especially the nuclease digestion unit operation, to ensure the adequate clearance of DNA impurities. With this data, we hope to highlight the key parameters which support optimal nuclease digestion to be applied to all viral vector processes, and thus streamline the work needed in process development for clearance of the DNA impurities.

1414 Intrathecal Lentiviral Gene Therapy to Treat X-linked Adrenoleukodystrophy Disease

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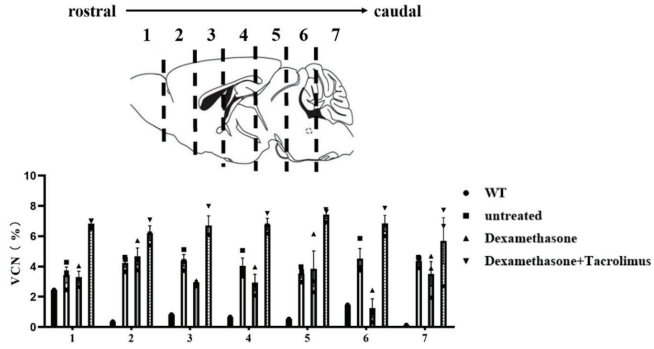
Introduction: X-linked adrenoleukodystrophy (X-ALD) is a genetic disorder due to mutation in the *ABCD1* gene involving defective peroxisomal β-oxidation of very long-chain fatty acids (VLCFAs). The defect leads to the accumulation of VLCFA in organs and tissues, which results in demyelination of central nervous system (CNS) and adrenal cortical atrophy or dysplasia. The current treatment for ALD is hematopoietic stem cell transplantation (HSCT). However, most patients cannot find matched donors, and HSCT is not effective for patients who have already developed symptoms. Gene therapy is a promising treatment for ALD. We hypothesize that direct intrathecal (i.t.) injection of lentiviral (LV) *ABCD1* gene may repair the deficiency. To prevent *in vivo* rejection, an immunosuppressive conditioning treatment was investigated before i.t. injection.**Methods:** An intracerebral LV-*ABCD1* injection gene therapy strategy in dl3/9*ABCD1* KO X-ALD mice has been developed (doi: 10.1038/s41434-022-00355-0). Here we explor an i.t. LV injection technique; each mouse received 50 ul of LV-*ABCD1* (transduction unit $2.3x10^8$) with or without dexamethasone and tacrolimus pre-treatment.**Results:** To establish the feasibility of i.t. LV injection, we first injected trypan blue dye into the fifth and sixth lumbar vertebrae of the wild-type (WT) mice. The results showed the diffusion of dye in both the brain and spinal cord. We next investigated the biodistribution of LV-GFP in WT mice by i.t. injection. The mice were injected with 50 ul of LV-GFP and the vector copy number (VCN) was examined in the peripheral blood (PB), and brain which was divided into 7 equally spaced parts from the rostral to the caudal (S1 to S7), and the spinal cord was divided into cervical, thoracic, and lumbar medulla. The *in vivo* distribution of LV-GFP was determined by q-PCR after 14 days. We detected VCN in three groups of mice: 1) untreated, or pretreated with 2) dexamethasone, 3) dexamethasone plus tacrolimus, and detected VCN in PB, cervical, thoracic, and lumbar medulla as the following: 1) 4.67%, 3.29%, 15.43%, 4.1%; 2) 6.04%, 6.34%, 5%, 11.2%; and 3) 6.45%, 4.95%, 10.07%, 8.13%, respectively. Further, we detected VCN in the brain regions (S1 to S7) of the three groups of mice: untreated, dexamethasone or dexamethasone and tacrolimus treated, as the following: S1) 3.44%, 3.30% and 6.83%; S2) 4.25%, 4.67% and 6.20%; S3) 4.39%, 2.95% and 6.37%; S4) 3.44%, 3.30% and 6.83%; S5) 3.55%, 3.85% and 7.43%; S6) 4.52%, 1.75% and 6.84%; and S7) 4.36%, 3.51% and 5.69%, respectively (see Figure). The above results showed that the LV-GFP could be distributed in the brain, spinal cord, and PB in the untreated group, and there were no significant differences compared with the other groups. Therefore, no pretreatment was done. We injected 2-month-old dl3/9ABCD1 KO mice in foramina of the fifth and sixth lumbar vertebrae with 50 uL of LV-*ABCD1* (treated group) or saline as control. The *in vivo* distribution of LV-*ABCD1* was determined after 22 days. The VCN was examined in the brain, spinal cord and PB as described above, and phenotype correction was evaluated based on established methodology.**Conclusion:** The i.t. injection of LV-GFP *ABCD1* demonstrated transgene biodistribution throughout the CNS as well as the PB and spinal cord *in vivo*. Further characterization of *ABCD1* expression and phenotype correction of i.t. LV-*ABCD1*injection in the dl3/9ABCD1 KO mice will be presented.



1415 Development of Oncolytic Adenovirus-Based Vaccine for Personalized Treatment of Malignant Mesothelioma

Jacopo Chiaro[1], Gabriella Antignani[1], Sara Feola[1], Michaela Feodoroff[1], Beatriz Martins[1], Hanne Cojoc[2], Valentina Ferrari[3], Daniele Ciampi[3], Ilkka Ilonen[4], Jari Räsänen[4], Mikko Mäyränpää[4], Jukka Partanen[5], Satu Koskela[5], Lukasz Kuryk[2], Salvatore Russo[1], Manlio Fusciello[1], Firas Hamdan[1], Maria Rescigno[3], Mikaela Grönholm[1], Rui Branca[6], Janne Lehtiö[6], Vincenzo Cerullo[1]

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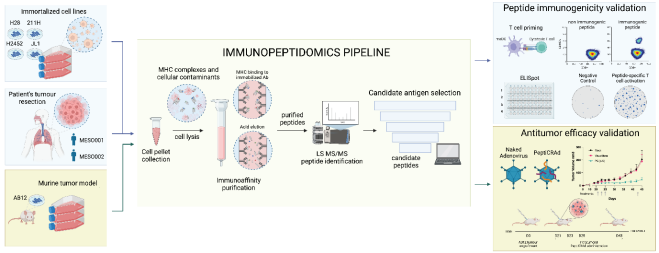
Malignant pleural mesothelioma (MPM) is an aggressive tumor with a poor prognosis. As the available therapeutic options show a lack of

efficacy, novel treatments and therapeutic targets are urgently needed. It has been observed that MPM is responsive to immunotherapeutic cancer treatments, and given its T-cell infiltration, we hypothesized that MPM is a suitable target for therapeutic cancer vaccination.To date, research on mesothelioma has focused on the identification of molecular signatures to better classify and characterize the disease, and little is known about therapeutic targets to engage cytotoxic (CD8+) T cells. In this study, for the first time, we explored the immunopeptidomic antigen-presented landscape of MPM in both murine (AB12 cell line), human cell lines (H28, MSTO-211H, H2452, and JL1), and in patients' primary tumors. Applying state-of-art MHC immuno-affinity purification methodologies, we identified MHC-I-restricted peptides presented on the surface of malignant cells. We characterized some newly discovered patients-derived peptides utilizing in vitro co-culturing techniques, demonstrating that eluted peptides showed promising immunogenicity profiles. Additionally, we provided a proof-of-concept for the application of a whole antigen discovery pipeline using immunopeptidomics for cancer vaccine development in a murine model of MPM. Overall, we investigated the antigen landscape of MPM and showed that the discovered peptides show potential to be used for therapeutic cancer vaccine. (This work is currently under revision in Nature Communication)



1416 Identification of Candidate Biomarkers for Type 1 Diabetes Mellitus by Bioinformatics Analysis of Pooled Microarray Gene Expression Datasets in Gene Expression Omnibus

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Background: Type 1 diabetes (T1DM) is a serious threat to childhood life and has a complicated pathogenesis. Currently, molecular mechanisms of T1DM remain largely unclear. The aim of this study was to identify the candidate genes in T1DM by integrated bioinformatics analysis. **Methods:** Transcriptomic datasets (GSE156035) in the GEO database were analyzed for differentially expressed genes (DEGs) using the R statistical language. The differentially expressed genes (DEGs) were identified, and the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed. A protein - protein interaction (PPI) network was applied to screen out the candidate genes. **Results:** The results revealed that 273 DEGs of the three datasets were ascertained in our study, including 135 upregulated genes and 138 downregulated genes. The GO and KEGG enrichment analysis results showed that the functions of DEGs mainly involved in regulation of transcription from RNA polymerase II promoter, specific granule lumen, transcriptional activator activity, Osteoclast differentiation pathway, etc. Through the PPI analysis network, the core genes with the highest degree of 6 nodes were selected: FOS, RHOA, CXCL8, FOSB, EGR1, and DUSP1. **Conclusion:** The genes, identified in this study, may play a vital regulatory role in the occurrence and development of T1DM. Also, they are closely related to obesity and diabetes mellitus. Our results provide novel biomarkers that could be used as representative reference indicators or potential therapeutic targets for T1DM clinical applications.

1417 Scalable Ultrafiltration-Diafiltration UFDF Process of Clarified Plasmid DNA pDNA Using TSeries Cassettes with Omega Membrane

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Plasmid deoxyribonucleic acid (pDNA) is an important genetic engineering tool used to clone and amplify or express genes for a wide range of applications. Production of viral vectors and messenger ribonucleic acid (mRNA) are dependent on pDNA. With the emergence of SARS-CoV-2, the development of novel mRNA/pDNA-based vaccines sped up significantly. The manufacturing process for pDNA poses challenges for process development and for regulatory authorities tasked with assuring quality, efficacy, and safety of the final product. For each process, the design of a suitable purification and concentration strategy will depend on many variables including the pDNA interaction with filtration module and the nature of the pDNA. Following the clarification step, the main objectives of the UF/DF step are to concentrate and buffer exchange the pDNA prior to a chromatography step. One of the challenges for UF/DF step involves plasmid compaction promoted by use of high salt buffers. This compaction can result in pDNA passage through the UF membrane, resulting in low yields. Critical process parameters during these studies were predetermined to avoid plasmid damage due to shear stress. Membrane fouling was prevented, and an effective recovery method was performed, both to avoid yield loss. We have developed a process that confirmed high retention of pDNA, increasing the pDNA yield post-clarification. A Pall Centramate™ cassette with Omega™ polyethersulfone (PES) membrane with a nominal molecular weight cutoff (NMWC) of 100 kiloDaltons (kDa) was used to concentrate and diafilter a clarified pDNA material with ~5 kilobase pairs (kbp) in a high salt buffer and resulted in significant product loss through the membrane. However, using a Pall Centramate cassette with Omega PES membrane with a NMWC of 30 kDa with a plasmid of similar size (~5 kbp), an initial volume concentration factor (VCF) of 10X, a 5 diavolume (DV) buffer exchange and a final concentration to an overall 15X VCF were achieved with no appreciable loss of pDNA product. Both runs were operated at a transmembrane pressure (TMP) of 12.5 psig (860 mbar). The average permeate fluxes for the Omega 30 kDa membrane in Centramate cassette format for the first concentration, the diafiltration, and the final concentration were 80, 80, and 85 LMH, respectively. The UF/DF step achieved the desired buffer conditions for chromatographic loading, provided adequate stability for the in-process intermediate, and had a >95% total pDNA recovery. The development studies presented here confirm Pall UF/DF technology as a highly efficient process solution for pDNA processing after clarification.

1418 Preclinical Specificity and Activity of CABA-201, a Fully Human 41BB Containing CD19 CAR T Therapy for Treatment-Resistant Autoimmune Disease

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Over 4% of the world population is estimated to live with autoimmune disease. Treatment typically requires systemic immunosuppressive therapy that have associated toxicities and are not curative. There is increasing evidence that B cells play a central role in disease pathogenesis, based upon responsiveness to B cell depletion by antibody-based therapeutics; however, responses are typically transient due to the incomplete depletion of B cells in secondary lymphoid tissue. Chimeric antigen receptor (CAR) T cells are a novel gene-engineered cellular immunotherapy where a synthetic T cell receptor is expressed to redirect the T cell to a desired target. Several B cell targeted CD19 CAR T cell products have led to durable remissions of B cell leukemias and lymphomas; three have been approved by regulators globally, each of which utilizes the murine derived CD19 scFv binding domain FMC63. Data from numerous studies have established the ability of these products to deeply deplete B cells. An early proof of concept pilot study evaluating the safety and efficacy of an FMC63-41BB-CD3ζ CAR T cell product, analogous to one of the approved therapies, in 5 patients with treatment refractory systemic lupus erythematosus suggests the potential to achieve rapid, deep and durable drug-free remissions. We designed a new CD19 CAR T product (CABA-201) containing a clinically de-risked (NCT05091541) fully human CD19 binder (IC78), to minimize immune mediated interference with activity. In addition, the construct utilizes the same 41BB costimulatory domain used in the pilot study above, which is reported to have a reduced incidence and severity of cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) in oncology patients. Preclinical studies were conducted to explore the specificity and activity of CABA-201 compared to the specificity and activity of the FMC63-41BB-CD3ζ construct using the same cell production method. CABA-201 demonstrated comparable cytotoxic activity to FMC63 CAR T cells against $CD19^+$ Nalm6 cells in vitro, and comparable in vivo potency was observed in a dose ranging study in the NSG-Nalm6 tumor model. No evidence of off-target cytotoxic activity of CABA-201 was identified against a panel of selected primary human cells, and no off-target binding against IC78 was detected in a membrane proteome array, or in clinical studies evaluating IC78 in a tandem CAR formation. CABA-201 generated from primary T cells from multiple autoimmune disease patients showed robust CAR surface expression and effective elimination of target autologous $CD19^+$ B cells in vitro. Together, these data support the safety and activity of CABA-201, and provide a clinically relevant benchmark for dose related potency in clinical studies planned for initiation later this year.

1419 Engineered Cas13 with Minimal Collateral Effect and Improved Editing Efficiency for Targeted RNA Degradation

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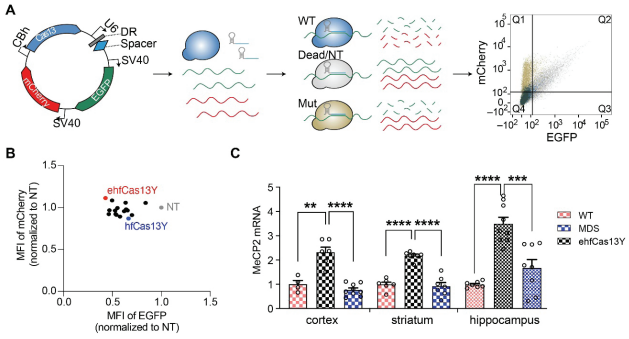
Background: The CRISPR-Cas13 family, showing high efficiency and specificity in programmable RNA targeting, has been widely used, and it is a promising approach for transcriptome engineering in basic research and therapeutic applications. However, collateral effects, for example, Cas13 protein degrades bystander RNAs once activated by interaction with the target RNA, have recently been found in drosophilas, mammalian cells, and mammals, hindering *in vivo* application. Previous structural studies have identified the mechanisms underlying collateral RNA degradation. However, to date, it's not clear whether there are any distinct binding sites for the target and nontarget RNA substrates near the catalytic site of activated Cas13 protein. **Methods:** To eliminate the collateral effects of Cas13, we constructed an unbiased screening system with EGFP, mCherry, and an EGFP-targeting gRNA together with each Cas13 variant in one plasmid and used FACS to select variants with high-efficiency on-target degradation (based on low EGFP fluorescence) and low collateral degradation activity (based on high mCherry fluorescence) (Fig. 1A). Based on structural analysis and biochemical characterization of Cas13, we hypothesized that changing the RNA-binding cleft proximal to the RxxxxH catalytic sites in the HEPN domains would selectively reduce promiscuous RNA binding and collateral degradation while maintaining on-target RNA degradation. Thus, we generated a mutagenesis library of Cas13 variants, each containing different one or more random amino acid substitutions (replacing all non-alanine with alanine, X>A, and alanine with valine, A>V). **Results:** After transfecting into HEK293 cells, we obtain several Cas13 variants that exhibited significantly reduced collateral activity, including hfCas13d, hfCas13X, and hfCas13Y, through FACS. The collateral effects revealed by transcriptome-wide RNA sequencing and cell proliferation analysis were also substantially eliminated. However, their endogenous RNA degradation efficiency was also reduced. For better application *in vivo*, we introduced the mutations showing improved cleavage efficiency, into the hfCas13Y protein and finally obtained a new hfCas13Y mutant (ehfCas13Y) with higher efficiency while ensuring low collateral activity (Fig. 1B). To explore *in vivo* therapies, we injected AAV viral particles comprising ehfCas13Y and gRNA targeting human *MECP2* mRNA into a humanized MECP2 duplication syndrome (MDS) mouse model. After 5 weeks, *MECP2* mRNA in the cortex, striatum, and hippocampus is knocked down to close to almost wildtype levels (Fig. 1C). **Conclusions**: The ehfCas13Y protein with minimal collateral effect and high efficiency has great potential for basic research and therapeutic applications for targeted degradation of RNA.



1420 Barcoded Mapping of Promoter Specificity and Efficiency in Human Brain Explants and the Mouse Brain

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Effective AAV gene therapies warrant a precise combination of capsid and promoter choice to tune therapeutic gene expression and limit off-target effects. To date, selection of AAV capsid and promoter pairs has relied on largely empirical data obtained by evaluating different vectors in preclinical animal models. We generated a barcoded promoter panel driving reporter transgenes and packaged within AAV9 for characterization in murine and human brain tissue. Briefly, the 22 promoters encompass a panel representing ubiquitous promoters, Herpesvirus (HSV)-derived latency associated promoters, as well as 5' upstream sequences of CNS specific genes tagged with barcodes for high throughput RNA sequencing. Following intracerebroventricular injection in mice we observe differences in promoter strength across five distinct brain regions. The Chicken Beta-Actin (CBA) promoter drives the strongest expression compared to Elongation factor 1 alpha (EF1a) and Cytomegalovirus (CMV) promoters. The 5' upstream sequences of human Neuron Specific Enolase (NSE) and S100-beta (S100B) drive highest transcript expression amongst the promoter panel in the mouse neurons and astrocytes, respectively. Further, enabled by an IRB approved protocol following patient surgery for intractable epilepsy, we evaluated the promoter panel in patient-donated, resected cortical tissue. High throughput sequencing data corroborated NSE and S100B as well as HSV-derived promoters for driving strong transcript expression in corresponding cell types. Further, we rank-order promoters of different strengths in both murine and human tissue, providing a roadmap for tailoring promoter selection in potential therapeutic applications.

1421 To Clear or Not to Clear: Interaction between Senescent Cells and the Immune System in HSPC Gene Therapy

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Preclinical and clinical studies of hematopoietic stem and progenitor cell (HSPC) gene therapy (GT) have shown that the integration of retroviral vectors nearby cancer genes may result in their deregulation, eliciting malignant transformation. Moreover, insertional activation of the *Braf* oncogene by genotoxic vectors in *Cdkn2a*$^{-/-}$ mice resulted in histiocytic sarcoma. BRAF is also known, in its V600E mutated form, to induce hematological disorders in humans (Langerhans Cell Histiocytosis, Erdheim-Chester Disease). We previously showed that BRAFV600E, expressed in human HSPCs transplanted in immune-deficient (NSG) mice, provoked oncogene-induced senescence (OIS). OIS was characterized by cell cycle arrest and aberrant secretion of pro-inflammatory cytokines as a result of the senescence-associated secretory phenotype (SASP). SASP ultimately triggered multi-organ infiltration of histiocytes, lymphoid impairment, and lethal bone marrow failure. Therefore, OIS acts as a double-edged sword: indeed, the secreted SASP factors attract immune cells, favoring the clearance of senescent cells (SCs). However, the chronic release of SASP may generate an inflammatory microenvironment, increasing the risk of comorbidities. Thus, OIS in the context of HSPC GT may pose risks before malignant transformation, by impairing immune cells, inducing oligoclonality, and other signs of early aging of the hematopoietic system. To study the interaction of SCs with immune cells and to identify mechanisms of resilience or clearance of oncogene-expressing cells, we investigated the fate of SCs in the presence or absence of an active immune system. We transplanted wild-type (WT) or NSG mice respectively with WT mouse (m) HSPCs transduced with lentiviral vectors expressing mBrafV600E, an N-truncated version (mBraf trunc), or GFP as control. In NSG recipients, both mBraf trunc and mBrafV600E expression caused dose-dependent lethality, the latter leading to a more aggressive phenotype. Multi-organ histiocytic infiltration occurred in 92% of mice of the mBrafV600E group and 30% of the mBraf trunc group. Instead, in WT recipients, mortality was reduced (only 60%). FACS analysis on peripheral blood and bone marrow samples showed reduced cellularity in both recipients, in particular in B and T cells, with higher impairment in NSG recipients. Interestingly, surviving WT mice of the mBrafV600E group were able to clear SCs, while NSG mice were unable to do so. At the base of this, we observed exacerbated blood plasma levels of CCL-2,-3,-4,-5, IL -2, -6, and -12 in NSG recipients, while in WT recipients only CCL-3,-4, and -5 were increased. Furthermore, transcriptional profiling of sorted myeloid and B cells in NSG and C57 recipients showed different signatures. In NSG recipients, mBrafV600E expression resulted in upregulated TNFα signaling, oxidative stress pathways, apoptosis, and unusual cell cycle inhibitor genes (*Cdkn2d* and *Cdk2ap2*). Downregulated processes were ribosome biogenesis, interferon signaling, MHC class II, and B cell activation. In C57 recipients, in contrast, B cells showed upregulated hallmarks related to cell cycle, DNA repair, and downregulation of apoptotic signaling. This study shows that the immunological background of recipient

mice may favor or impair the efficient elimination of SCs. Future experiments will dissect the specific subset of immune cells responsible for the clearance, with the use of recipient mice with different genetic backgrounds. In the context of HSPC GT, often dealing with immunocompromised patients, our final goal is to identify mechanisms for the elimination of SCs to improve the safety of GT strategies.

1422 Preclinical Trials of an rAAV-Dysf for LGMD R2. Preliminary Results

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Introduction. Limb-girdle muscular dystrophy type 2B and Miyoshi myopathy, which are among the most prevalent muscular dystrophies, are caused by mutations in the DYSF gene. There is currently no effective treatment for these diseases. AAV vector gene transfer is a promising approach to treat muscular dystrophies. In this work, we present early preclinical data of AAV-Dysf trials. Methods. In vitro experiment was performed on dysferlin-deficient artificial myoblasts. In vivo experiment was carried out on a sample of 96 mice, 64 of them received AAV-Dysf IM and IV. Muscle samples were obtained from 32 Bla/J (AAV-Dysf) mice 3 months post-delivery. The transgene delivery was confirmed using PCR, RNA detection by RT-PCR, and protein detection by ELISA and immunohistochemistry. A histopathological study with morphometry was performed to assess the necrotic muscle fibers, the proportion of the centrally located nuclei in muscle fibers, and the average cross-sectional area. Physiological tests were performed. Results. All samples were registered with the transgene cDNA, dysferlin mRNA, and dysferlin protein. Also, there were changes in the structure of muscle tissue with regards to the proportion of necrotic, central nuclear muscle fibers and the average cross-sectional area of muscle fiber. Conclusions. It was demonstrated that intravenous and intramuscular administration of a dual AAV vector system carrying 5' and 3' cDNA fragments of the dysferlin gene was able to restore a full-length dysferlin protein, we have registered improvements in physiological tests. In addition to that, muscle tissue structure showed no obvious toxicity.

1423 Non-Replicating, Non-Integrating Vector for Immunotherapy and Vaccine Applications

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Non-replicating, non-integrating vectors can provide transient expression in proliferating cells and stable transgene expression in quiescent cells. We explored further engineering of our highly modified non-replicating Moloney Murine Leukemia Viral (MoMLV) vector to become non-integrating using our optimized Herpes Simplex Virus Thymidine Kinase (HSV-TK) gene construct as a model system. The optimal vectors for gene delivery should exhibit large payload capacity, high transduction efficiency, target cell selectivity, and no genotoxicity. For cancer therapy and vaccine use, an acceptable immune response is also necessary. Non-integrating vectors will dilute progressively in proliferating cells. However, for certain gene products, transient expression may be sufficient. Additionally, non-integrating vectors in particular offer a reduced risk of genotoxicity. For vaccine applications, transient expression may also be useful. If stable expression in non-dividing cells is required, repeated administration of non-integrating vectors may be considered. In order to generate a non-integrating vector, seven integrase mutants were constructed at the Mg^{2+} binding motif of the catalytic core domain of the integrase in the vector *gagpol* gene: single mutations, dual combinations, and a triple mutant. The constructs were tested by triple transfection of 293T cells with *env*, *gagpol*, and *payload*. None of these mutations affected the physical titer of vector. Using our optimized HSV-TK suicide gene construct as the payload, the level of protein expression was examined in melanoma A375 cells transduced with vectors made with *gagpo*l containing the integrase mutants and compared with payload expression from vectors made with wild-type *gagpol*. Data from FACS and Western blots for HSV-TK protein from the various mutants indicated that early protein expression is strong and nearly comparable to that observed with a functional integrase. This expression correlates well with the cell kill activity in the presence of ganciclovir (GCV). As expected, expression decreased over time to undetectable levels as the cells divided. Lack of integration in the host genome was confirmed using relative integration qPCR. All mutations and their combinations were able to knock out host genome integration. A range of cancer cell lines were tested with the non-integrating vectors. When the cells were successfully transduced with the vectors, HSV-TK expression became undetectable between 7 and 14 days for most of the cell lines tested. This correlated with the loss of cell kill activity in the presence of GCV. To examine the functional effect of the mutated integrase with the reverse transcription deficiency, the reverse transcriptase (RT) gene was also mutated in the *gagpol.* When the mutated RT construct was used in the triple transfection of 293T, the vector particle was produced at a high titer, but transduced A375 cells failed to express the HSV-TK protein. No GCV induced cell killing was observed as well. Not surprisingly, when a combination of integrase mutated and RT mutated vector was tested, expression of the payload was also not present. This implies that RT activity is required even in the non-integrating vector, and that payload RNA alone is insufficient in transduced A375 cells for detectable expression of HSV-TK protein. With non-replicating, non-integrating vectors, there is the opportunity to explore many additional indications in the immunotherapy and vaccine areas. An example is incorporating both an antigen gene and a second gene, e.g., a viral thymidine kinase. Incorporation of both of these genes within a non-replicating, non-integrating vector would permit the combination of antigen production with subsequent GCV-mediated cell killing, with the potential to augment the immune response against the co-delivered antigen.

1424 Development of an Innovative, Scalable, High Productivity Manufacturing Process to Enable Phase I-II Clinical Supply of UX810, an Investigational AAV Gene Therapy for the Potential Treatment of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked recessive, neuromuscular disorder characterized by muscle weakness and atrophy leading to significant developmental motor milestone delays, respiratory and heart failure and premature death. Treating rare neuromuscular genetic disorders with gene therapy requires a significantly higher dose than most other target organs because systemic delivery of product requires widespread distribution to target as many muscle cells as possible. Recombinant adeno associated virus (rAAV) is the leading platform for gene delivery for numerous rare disease therapies, however; high-dosing requirements and low batch yields lead to significantly high costs of manufacturing. There is an urgent need to improve existing rAAV manufacturing platforms to achieve robust, high-yielding, scalable and cost-effective processes to meet high dosing requirements, patient demand, and reduce the overall cost of treatment in order to make therapy accessible to patients. Ultragenyx has developed a cost-effective and scalable rAAV production Pinnacle producer cell-line adenovirus helper platform that eliminates the high cost of goods (COGs) associated with transfection-based processes. In this study, improvement over the legacy batch platform process was demonstrated using intensified manufacturing approaches to achieve greater than 3- fold increase in overall volumetric rAAV yield. Process intensification increased rAAV platform productivity near 1 x10^{12} GC/mL (~1 x10^{15} GC/L) at 250 L scale to enable program development for treatment of DMD. COGs analysis demonstrates this novel platform significantly reduces number of cGMP batches required to initiate Phase I-II clinical studies compared to the legacy process at a significantly reduced cost while meeting high dosing requirements for DMD treatment (Figure 1).

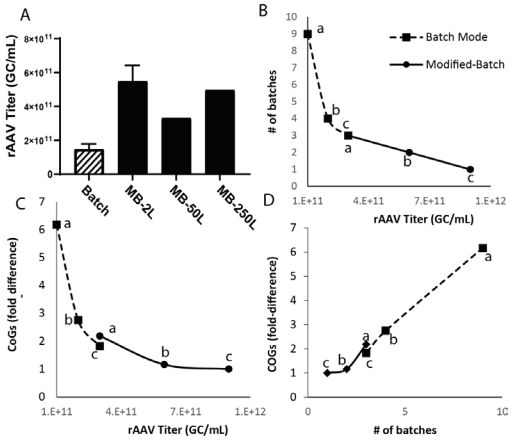

Figure 1. Modified-Batch Yield and COGs Projections for PhI-II DMD Clinical Trial Demand

A) Modified-Batch (MB) rAAV upstream process titer (GC/mL) compared to batch process titer at 2L, 50L, and 250L production shows greater than 3-fold boost in volumetric yield and consitent scaleable performance. **B)** Projection of # of batches needed based on upstream Process Yield for 3 model clones with various specific productivity (a-Low, b- Med, c-high) to support Ph I-II Clincal Trials for DMD using either batch-mode rAAV platform (dashed) vs. new modified-batch perfusion process (solid). **C)** Fold-difference in CoGs projections based on upstream rAAV volumetric yield (GC/mL) for batch (dashed) and modified-batch perfusion process (solid) and **D)** COGs vs. # of batches.

1425 The PS Gene-Editing (PSG) System for Treatment of Lysosomal Diseases

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Background. The PS Gene-editing (PSG) System is a novel CRISPR-based gene editor that enables lifetime production of therapeutic proteins systemically. The PSG platform has proven safe and efficacious in validated murine models of 4 lysosomal diseases: mucopolysaccharidosis (MPS) type I, Sandhoff and Tay-Sachs diseases, and GM1-gangliosidosis. Program Plan. Toward this goal, the PSG System will be transitioned from AAV delivery to a new formulation of lipid nanoparticles (LNP). In so doing, PSG eliminates the potential for an immunogenic response to capsid epitopes, and eliminates the barrier of pre-existing immunity to AAV, allowing (i) universal targeting regardless of innate immunity or prior exposure to AAV; (ii) repeated dosing; (iii) increased flexibility of payload size; and (iv) anticipated future use for in utero therapy. The LNP delivery vehicle also promises faster production with scalable, vetted GMP-compatible equipment, formulations and processes at reduced cost. In addition, it offers malleable surface characteristics for flexible cell-surface targeting. This program will also implement innovative technology to enhance the PSG gene-editing payload to improve on-target specificity and reduce the risk of off-target genomic insertions. Summary. Since generating the essential preclinical results and participating in the clinical trials (NCT02702115 and NCT03041324) of the first-ever human in vivo gene editing (13-NOV-2017), this program is now at the nexus of (i) decades of bench research and clinical treatment of MPS diseases and other lysosomal conditions; (ii) decades of experience with gene therapy; (iii) evolution of a novel "gain of function" CRISPR-Cas9 PSG System gene editing technology program; and (iv) the cresting wave of LNP technology.

1426 Accurate Integration of piggyBac Transposon into Defined Genome Locus by ACTinG

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Gene editing has achieved much advance in the past decade, especially the development of CRISPR-Cas system and its derived tools such as base editors and prime editors. These game-changing tools allow precise gene editing in genome and transform the cell & gene

therapy field. However, precise integration of a large DNA fragment into a specific site of mammalian genome remains challenging. The piggyBac (PB) transposon is widely used to efficiently integrate large cargo into mammalian genome in the "TTAA" dependent manner. PB is almost randomly integrated as it displays little selectivity for particular genome regions. Excision competent yet integration defective (Exc+Int-) PB mutants have little "TTAA" dependent integration. Fusion of Exc+Int- PB mutants to Cas9 takes the advantages of the precision cleavage of Cas9 and the high integration efficiency of PB. We have developed several Exc+Int- PB mutants with high excision but little integration activity in comparison with the WT PB. Fusion of these mutants to Cas9 results in a brand new tool for ACcurate Transposon into Genome (ACTinG) with double strand breaks generated by Cas9-sgRNA cleavage. This tool can efficiently integrate transposon carrying a large cargo (up to 10kb) into a precise genome locus. The integration efficiency is about 4-8 folds higher than that of previous reported Cas9-PBase (R372A_K375A_D450N) and HDR methods. The on-target integration efficiency can be even higher as much as 60% - 80% with a modified donor sequence. These results demonstrate that ACTinG is a robust and efficient tool for precise integration of large DNA cargos into mammalian genome. Further characterization and optimization of this gene editing tool will potentially increase its therapeutic value in different areas.

1427 Bioengineered AAV3B Capsid with Improved Human Hepatocyte Tropism and Evasion of Pre-Existing Neutralizing Antibodies

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Adeno associated virus (AAV) vectors are versatile gene delivery vehicles due to their low genetic complexity. Ongoing research is focused on lowering the overall vector dose, eluding pre-existing neutralizing antibodies (NAb) and improving tissue tropism. Previously, we generated an AAV3B combinatorial capsid library by integrating rational design with directed evolution and identified an isolate, AAV3-DE5, with significantly improved hepatotropism over 5 rounds of iterative selection in hepatocyte spheroid cultures. In this work, we reanalyzed the original dataset to isolate variants from earlier (1-3) iterations based on the assumption that variants with faster replication kinetics might not necessarily be the most efficient at transducing the cell of interest. We identified two new variants: V04 (mutation in VR-IV) and V05 (mutations in VR-IV to VR-VII) with an enrichment score greater than 1. The region from amino acids 351-570, which include mutation regions VR-IV through VR-VII was then synthesized and cloned into the parent AAV3B capsid backbone. In vitro, AAV3B-V04 transduced human hepatocellular carcinoma (HUH-7) cells or mouse passaged primary human hepatocytes (mpPHH) at significantly greater frequencies as compared to AAV3B, AAV3V-DE5, or AAV3B-V05, indicating improved hepatotropism. Similarly, in vivo, AAV3B-V04 transduced human hepatocytes in huFNRG mice at significant frequencies as compared to other engineered variants such as AAV3B-ST or AAV3B-DE5, with high detargeting of mouse hepatocytes. Human hepatocyte transduction efficacy in huFNRG mice was 5-14% for AAV3B-ST, 10-27% for AAV3B-DE5, and 16-35% for AAV3B-V04. Next, we tested the effect of pre-existing NAbs on the efficacy of AAV3B-V04 transduction using IVIg or individual serum samples from healthy donors. The average IVIg concentration required to neutralize transduction by 50% (IC_{50}) for AAV3B, AAV3B-DE5 and AAV3B-V04 was 38, 115.6 and 256 μg/mL respectively, with an observed 6.7-fold- increase in IC_{50} for AAV3B-V04 over the parent AAV3B capsid. Of the 30 human serum samples with detectable (>1:5) NAb titers to AAV3B and AAV3B-DE5, we observed a substantial reduction in reciprocal NAb titers, that were on average 4-fold and 3-fold lower for AAV3B-V04 as compared to AAV3B or AAV3B-DE5, respectively, indicating significant NAb evasion by AAV3B-V04. Of note, in 6 serum samples with undetectable NAbs to AAV3B or AAV3-DE5, we did not detect NAbs to AAV3B-V04, demonstrating that no new immunogenic epitopes were created in the library selection for AAV3B-V04. In summary, AAV3B-V04 demonstrates improved transduction efficiency and reduced immunogenicity as compared to the parent AAV3B capsid and previously described AAV3B-DE5 and AAV3B-ST variants. Since the liver is an important target for gene delivery in many gene therapy applications, AAV3B-V04 as a vector offers high potential to explore translational liver gene therapy studies

1428 Comparative Analysis of Second Strand Synthesis Processes to Establish an rAAV Batch Quality Pipeline Using Nanopore Sequencing

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During recombinant AAV (rAAV) production for therapeutic use in gene therapy, the vast majority of encapsidated DNA consists of the correct, full-length vector genome cassette or fragments thereof. However, undesired impurities derived from production plasmids or host cell genomic DNA can become encapsidated in a small but not insignificant proportion of capsids. The comprehensive analysis of encapsidated DNA is key for understanding safety and efficacy of an rAAV batch and to identify weak spots in the genome or in the manufacturing platform. Quantitative polymerase chain reaction (qPCR) or droplet digital PCR (ddPCR) remain the standard for DNA quantification of both vector genome and DNA impurities, but these methods require sequence specific primers that result in selective collection of information regarding encapsidated impurities. Furthermore, these methods provide only very limited information about functionality of DNA, such as length or sequence integrity. Next-generation sequencing (NGS) is an important advancement as it does not require *a priori* sequence information. Long-read NGS technologies, such as nanopore and single molecule, real-time (SMRT) sequencing, can provide reads of several kilo- to megabases originating from a single DNA molecule. Therefore, long-read technologies offer the unique opportunity to collect length and sequence integrity information on encapsidated single-stranded (ss) AAV genomes but also on other encapsidated impurities derived from the production platform. A remaining challenge is that current NGS techniques require double-stranded (ds) DNA as input for sequencing library preparation, whereas AAV genomes and packaged impurities are ssDNA molecules. Different

approaches have been described to convert these ssDNA molecules into dsDNA molecules. The simplest method is the extraction of encapsidated DNA and annealing of complementary (+) and (-) strands. However, efficient performance of this method depends on having abundant packaged (+) and (-) strand sequences for a given DNA segment, generating a bias towards reads of the vector genome cassette or major plasmid derived impurities. Minor impurities such as randomly packaged host cell DNA and/or plasmid-derived sequences might not anneal to form dsDNA required for sequencing adapter ligation and as a result are lost during sequencing library preparation. To this end, a dedicated second strand synthesis step is mandatory to characterize encapsidated DNA impurities by NGS in an unbiased way. We examined different processes to generate material that is amenable for NGS library preparation and tested it with the latest available nanopore sequencing chemistry with improved read quality. Results showed that a robust second strand synthesis can be achieved using random primers and different enzyme classes. We have performed control experiments using synthesized ssDNA without annealing potential to show that dsDNA is generated regardless of the sequence length and complexity. We also focused on the challenging structure of the inverted terminal repeats (ITRs) and elucidated their impact on the second strand synthesis process. This work highlights the importance of the dsDNA library preparation and quality control process to avoid generation of data artifacts that may lead to misleading conclusions.

1429 Targeted Integration to Endogenous Sites in the Human Genome Using CRISPR-Associated Transposases Discovered from Natural Environments

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Integration of DNA into targeted genomic sites has historically been a challenge for therapeutic gene editing. Established transposase and lentiviral systems are efficient at inserting large DNA cargos into the human genome, but result in non-specific and sometimes hyperactive integration. These integrations have resulted in severe adverse events during clinical trials in the form of neoplasia. CRISPR associated transposases (CAST) are a potential solution to this problem, as they could provide user-directed and programmable DNA integration. Although relatively rare in nature, the handful of known CAST systems are efficient at delivering large DNA payloads into bacterial genomes; however, translation to mammalian cells has not been possible. We hypothesized that novel CAST identified from metagenomic sequences of environmental samples would enable discovery of systems more amenable to use in human cells. Mining of millions of assembly-driven, metagenomic sequences from diverse environments uncovered active CAST capable of efficient transposition in vitro and into the E. coli genome. When delivered to mammalian cells, these CAST components are expressed in an active form and localized to the nucleus. When tested in cells, we reproducibly achieved programmable transposition into multiple endogenous sites in the human genome. Our results augur well for the development of CAST into tools for treatment of genetic disease.

1430 Development of PSMA-Targeted Oncolytic Adenovirus for Treatment of Prostate Cancer

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Prostate specific membrane antigen (PSMA) is highly expressed in poorly differentiated, metastatic, and castration-resistant prostate cancer. It is therefore reasonable to develop a PSMA-targeting therapy for prostate cancer treatment. Oncolytic adenovirus (OAd) is a promising candidate of cancer therapeutics, but many cancer cells are resistant to wild-type OAd infection due to the low expression of adenovirus primary receptor (coxsackie-adenovirus receptor, CAR). In addition, CAR binding of the OAd causes a variety of issues such as toxicity in and sequestration by CAR-positive organs (e.g. liver, lung). Vector retargeting is therefore necessary for adenovirus based transduction of most PSMA positive cancer cells. To generate the cancer-targeting OAd, we have previously established a system for isolating transductionally-targeted infectivity-selective adenovirus by Ad-based library screening approach. Here, we isolated the PSMA-targeted OAd by using *in vivo* Ad-based library screening, and tested the oncolytic activity of the PSMA-targeted OAd. PSMA-targeted OAd (PSMA-OAd) showed strong binding to PSMA-positive cells (HEK293-PSMA and CWR22R (PSMA (+) human prostate cancer cell line)), not to PSMA-negative cells (parental 293 and PC3 (PSMA (-) prostate cancer cell line)). In addition, the anti-PSMA antibody inhibited the binding of the PSMA-OAd, and also RNAi-based PSMA knockdown cells showed weak viral binding compared to a parental cells. For the oncolytic activity, the PSMA-OAd showed strong oncolysis selectively in PSMA-positive cells. In order to assess the *in vivo* function of PSMA-OAd, we assessed *in vivo* viral distribution after intravenous injection (i.v.). We measured the virus copy number in major organs (lung, liver, spleen, kidney, intestine, and tumor) after i.v. injection of the viruses. The PSMA-OAd showed reduced liver and lung targeting compared to untargeted OAd (Ad5-WT). Next, we tested the anti-tumor efficacy of PSMA-OAd. After intravenous administration of the viruses, only the PSMA-OAd showed significant antitumor effect compared to the untreated group ($p<0.05$), while the growth of Ad5-WT virus injected group was same as untreated group. In this study, PSMA-OAd exhibited selective infectivity and oncolysis to PSMA-positive cells, and also systemically injected PSMA-OAd showed remarkable anti-tumor effect. Our results suggest that the PSMA-OAd can embody efficient systemic treatment for metastatic prostate cancer.

1431 Assessment of Investigational Gene Therapy and Gene Editing Program Sponsor Compliance with Twenty-First Century Cures Act and 42 CFR Part 11 Reporting Requirements

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The 21st Century Cures Act, passed in 2016, established a requirement for US sponsors of investigational treatments to create public-facing statements regarding pre-approval access to their investigational product. Once Phase 2 trials are initiated, each company's policy for access must be made publicly available, typically on the sponsor's corporate website. Similarly, in 2017, under 42 CFR Part 11, a requirement for posting study results to the ClinicalTrials.gov website within one year of study completion was initiated. Because investigational Gene Replacement Therapies (GT) and Gene Editing (GE) programs generally produce less clinical trial material than small molecules and biologics, it is unknown whether these limitations of supply impact pre-approval access. Similarly, because long-term safety and durability of efficacy for GT and GE programs are typically assessed beginning in first in human trials, it is also unknown whether sponsoring companies report, or are capable of reporting, meaningful results to clinicaltrials.gov within one year of completion of dose-finding in Phase 1, First in Human (FIH) Studies. **Materials and Methods**: Structured electronic searches of the ClinicalTrials.gov trial repository for active and completed Phase 2 trials with investigational GT and GE products will be conducted a maximum of 60 days prior to presentation. For GT and GE programs in Phase 2 development, searches of all sponsoring company websites for statements regarding pre-approval access, will be conducted and results reported. Structured searches for all applicable and completed GT and GE trials noted in clinicaltrials.gov will be conducted and the presence or absence of posted results within 12 months of the posted completion date, in compliance with the 2016 and 2017 laws, respectively, will be determined. **Results**: Frequencies of investigational GT and GE trial sponsor compliance with the 21st Century Cures Act and 42 CFR Part 11 results reporting requirements will be summarized. Descriptive statistics will be reported for all non-categorical variables, and any relationships identified by therapeutic modality, indication, adult or pediatric age, rare/orphan status, study phase, and public or private funding. **Conclusions**: Proposed based on observed results.

1432 EVADER Reduces Immunogenic Response to AAV8-FIX Capsid

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We have previously shown that we were able to get greater gene transfer and Factor IX expression using our EVADER-AAV8-FIX treatment in mice and non-human primates. We have done further analysis on the NHP study by taking terminal (Day 78) PBMCs and performing ELISPOT analysis after stimulation of the isolated PBMCs with AAV8 peptides. At the 5E10 vg/kg dose level, there was a 7-fold drop in IL6 producing cells after a single dose and a 3-fold drop after two dose administrations when comparing standard AAV to EVADER AAV. At the 5E10 vg/kg dose level, there was a 3-fold drop in TNFα producing cells after a single dose and a statistically insignificant 1.3-fold drop after two dose administrations when comparing standard AAV to EVADER AAV. There was an even more dramatic drop in doubly positive IL6/TNFα cells of 11-fold at the single dose administration. There was a trend to a 5-fold drop in the double dose administration group (but not significant due to high variability). The enhanced effect of the doubly positive IL6/TNFα cells suggest a synergistic effect of these two cytokines. Further studies will sample PBMCs at different time points for higher resolution mechanistic analysis and may suggest ideal timing between dose administrations. Interestingly, it was shown that anti-capsid Nabs were not significantly reduced by EVADER despite the impact on cytokines as well as seeing additional vector genomes delivered with a second dose. Analysis revealed that huCTLA-4 content on EVADER vector used in the NHP study was dosed at 0.05 ng/kg which was 3 logs lower than muCTLA-4 levels on EVADER vectors in our mouse studies (which was dosed at 70 ng/kg). Abatacept, a soluble version of CTLA-4, is used to treat Rheumatoid Arthritis and reduces patient antibodies. Indeed, Takeda showed that Abatacept, co-administered with the same AAV8-human FIX vector used in our studies, significantly reduced anti-capsid antibodies. Therefore, increasing CTLA-4 levels on EVADER vector batches used for future NHP studies may prove beneficial. CTLA-4 levels on EVADER particles are related to the amount of CTLA-4 protein on the surface of producer cells. In its native role, CTLA-4 is internalized in T cells in response to T cell activation. In our experience, murine CTLA-4 used to make EVADER particles for our mouse studies did not appear to internalize. Human CTLA-4 however does seem to be internalized in HEK293 producer cells resulting in reduced human CTLA-4 on EVADER. Using different strategies, we were able to increase human CTLA-4 levels in EVADER by 2 logs by changing our manufacturing process.

1433 AAV-Mediated, Small Molecule-Riboswitch-Controlled Delivery of Growth Hormone Rescues Growth in GH-Deficient *B.little* Mice

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AAV-mediated gene transfer is a promising therapy for many diseases. However, excessive amounts of therapeutic protein—especially hormones or growth factors—expressed from unregulated vectors may result in unwanted side effects and a narrow therapeutic window, as well as limiting efficacy of gene therapy. Here, we present the development of precisely regulated human growth hormone gene, whose expression is controlled by a splicing-based riboswitch via small molecule inducer. Different from previously reported gene regulation systems that involve using exogenous protein components, our gene expression platform utilizes a riboswitch which is an RNA element that contains an aptamer as a sensor for a small molecule ligand/inducer. The riboswitch has been built for activity in mammalian cells and results in activation of gene expression from a very low or undetectable basal level in the absence of

the small molecule, to therapeutic levels in a tight dose response to the orally delivered small molecule. In the absence of the small molecule inducer *in vitro*, the growth hormone (GH) gene with the riboswitch does not express growth hormone protein. In the presence of the small molecule inducer, growth hormone is robustly produced in an inducer dose-dependent manner. When the growth hormone gene with riboswitch was delivered in AAV into the muscle of growth hormone deficient *B.little* mice via local intramuscular injection, the oral small molecule inducer treatment resulted in increased body weight and body length of the mice. The improvement of the animal growth in *B.little* mice indicates that the induction of expression of growth hormone achieves therapeutic level in these animals and demonstrates for the first time rescue of GH deficiency via the delivery of a small molecule inducer of a locally delivered gene therapy. Our data provide evidence that our riboswitch platform provides efficacious and safe gene therapy for delivering GH via gene expression control.

1434 LentiVEX™ Producer Cell Lines: Transfection-Free Production of Lentiviral Vectors Encoding Therapeutically Relevant Transgenes

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Lentiviral vectors (LVV) are of increasing interest in cell and gene therapy, but high costs and variable vector manufacturing process continue to limit patient access. We thus developed LVV producer cell lines that reduce vector manufacturing cost and process variability as they do not require plasmid preparation, testing or transfection for production of LVV. We first developed an LVV packaging cell line by random integration of Tet-regulated VSV-G and Gag-Pol, and constitutive Rev (codon optimised and regions of DNA homology deleted to decrease risk of recombination) into clonal WXATUS0028 suspension HEK 293 cell line, followed by clonal isolation of the top performing clone. We then developed an LVV producer cell line by transposon-facilitated integration of LVV genome into the top packaging cell line clone, followed by clonal isolation and performance screening. The top clonal producer cell line encoding EGFP produced LVV up to 4.6 $\times 10^8$ TU/mL. Furthermore, we trialled development of LVV producer cells encoding several different therapeutically relevant transgenes, and all producer cells yielded comparable or higher LVV titres than the four-plasmid transfection-based production. Further research work is ongoing to achieve transgene repression in producer cell lines to minimise any transgene impact on the producer cells and thus to maximise LVV titre. In summary, LentiVEX producer cell lines produce LVV at titres comparable to the industry standard four-plasmid method, and reduce manufacturing costs and process variability, improving patient access to LVV-based cell and gene therapies.

1435 Induction of Tregs in the Liver as a Result of Hepatic Gene Transfer is Unhindered by Chronic Autoimmunity

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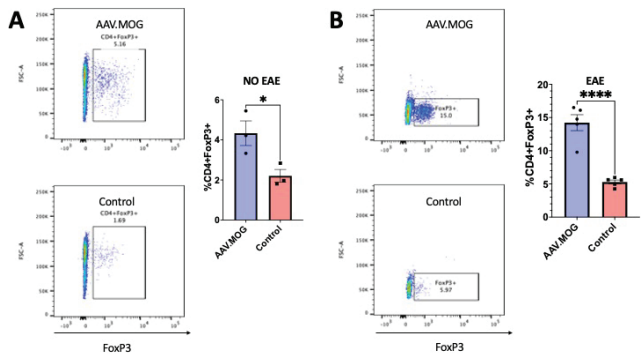
Adeno-associated viral (AAV) gene therapy has opened the door to treating a multitude of genetic diseases through its capability to target specific tissues for expression of a missing or corrected protein. Liver-directed gene therapy has been utilized as a method to treat Hemophilia B as well as the mouse model of Multiple Sclerosis, Experimental Autoimmune Encephalomyelitis (EAE); the latter of which is a result of the generation of transgene-specific $CD4^+FoxP3^+$ T regulatory cells (Tregs) capable of ameliorating disease. Previous work has showcased that early intervention of EAE with a viral vector carrying the sequence for the full-length myelin oligodendrocyte glycoprotein (MOG) reduces clinical and pathological severity of the disease; however, when this novel immunotherapy is given at the chronic/late stage of disease, there appears to be no therapeutic benefit. We hypothesize that Treg generation brought on by hepatic gene transfer has been abrogated as a result of EAE. Two experiments were conducted to determine the validity of this hypothesis. The first involved administering AAV.MOG or control to C57BL/6 (B6) mice and, after at least two weeks, harvesting their spleens and livers to analyze the frequencies Tregs within those organs. In the second experiment, B6 mice were afflicted with MOG_{35-55} EAE and were treated with AAV.MOG or control 30 days after disease induction. Treg frequencies in blood, spleens, and livers were examined two weeks after vector administration. Sections of liver were taken for Western Blot analysis from both experiments to confirm MOG expression. In mice that were not afflicted with EAE, there were no significant differences of Treg frequencies in the blood or spleens of both groups. In accordance with established literature, there was a significantly higher frequency of Tregs in the livers of mice given AAV.MOG as compared to the controls (Figure 1A). Interestingly, in mice that underwent EAE induction, Treg frequencies were significantly increased in both the livers (Figure 1B) and blood of AAV.MOG-vectored mice, indicating that EAE has not prevented that process. There was no significant difference of Treg frequencies in the spleens of either group. Clinical scores between the AAV.MOG and control groups showed no difference in EAE severity. In summary, EAE does not stop the induction of Tregs after liver-directed gene therapy as indicated by the significantly increased frequencies of $CD4^+FoxP3^+$ cells in mice treated with our therapy.



1436 Development of a Next-Generation AAV Helper Plasmid with Improved AAV9 Productivity

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rAAV vectors have proven to be safe and effective at delivering genetic material to patients. To date, rAAV has mainly been used to treat rare monogenic disorders. To broaden its use, current production methods must be improved to reduce costs and enhance functional titers. Many manufacturing platforms rely on transient transfection of a helper plasmid containing adenoviral genes E4, E2A and VA-RNA to mediate AAV replication. Whilst this construct can produce functional vector, little work has been done to optimize the combination of viral elements in order to maximize AAV production. We used the ATLAS (Arrayed Targeted Library for AAV Screening) platform to perform a high-throughput screen of a library of viral elements evaluating individualistic as well as combinational effects of these viral genes on AAV9 production. Targets from this screen were incorporated into our minimal helper construct and resulted in >5-fold increase in AAV9 production compared to the industry standard vector. We have confirmed this novel helper construct improves both the potency and yield of AAV9 with multiple cassettes. Studies are underway to evaluate the utility of this construct in multiple AAV serotypes and in a large-scale bioreactor system.

1437 Correction of Diverse Factor 8 Mutations by AAVHSC Genome Editing

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Hemophilia A (HA) results from diverse mutations of the Factor 8 (F8) gene, leading to an inability to form blood clots. Apart from approximately 45% of cases due to Intron 22 inversion (I22I), more than 3000 unique mutations of the F8 gene have been associated with HA with different degrees of severity. AAV gene transfer vectors delivering truncated and modified F8 transgenes have recently shown promising clinical efficacy in treating severe HA. However, one trial reported a decline in therapeutic efficacy over time, likely due to loss of persistence of episomal AAV vector genomes. Supraphysiologic levels of clotting in another trial led to thrombotic events. These outcomes emphasize the need to develop strategies for the stable physiologic expression of corrected genes. The permanent correction of genetic mutations by genome editing offers clear advantages over gene transfer therapy. The corrected genome is expected to provide persistent, long-lived therapeutic efficacy and regulated physiologic expression levels under the control of endogenous regulatory circuitry. AAVHSC represent a family of naturally occurring AAVs isolated from hematopoietic stem cells that mediate efficient homologous recombination-based genome editing in the absence of exogenous nucleases. We have previously demonstrated successful editing of the I22I mutation of the F8 gene using AAVHSC genome editing vectors in primary human and I22I patient-derived cells. The vector consisted of left and right homology arms flanking a codon optimized cDNA of exons 23-26. We have also demonstrated efficient and stable in vivo editing of the genomic F8 locus in mice. Here, we explore the potential of the AAVHSC platform to correct more than 90% of the remaining over 3000 mutations that are scattered throughout nearly 184 kb large F8 gene. To achieve this, we have designed three editing vectors to correct mutations located throughout the F8 gene. The N-terminal editing vector consisting of a codon optimized exon 2-13 cDNA is designed to correct all mutations in those exons. Upon successful editing, the codon optimized cDNA is designed to replace the endogenous genomic sequences spanning exons 2 to 13. The transcript from the edited F8 gene will then be spliced to the downstream exons 14 to 26 to generate a full-length mRNA encoding the wild type FVIII protein. Similarly, a central editing vector consists of a codon optimized exon 14 flanked by homology arms. Similarly, the C-terminal editing vector consists of the codon optimized cDNA of exons 15-26 flanked by homology arms. Unlike the N-terminal and the central editing vectors, the C-terminal editing vector mediates targeted insertion at the start of exon 15 and does not replace a large segment of F8 genomic DNA. Notably, this allows retention of several important genes that are encoded within the 3' end of the F8 gene. These vectors were packaged in AAVHSC15 and used to edit primary human liver sinusoidal endothelial cells, the primary producers of FVIII protein. Successful editing was observed in 10-40% of cells with the N-terminal and C-terminal editing vectors. Together these data demonstrate the feasibility of therapeutic correction of over 90% of diverse non-I22I F8 mutations associated with hemophilia A.

1438 Multi-Epitope DNA Vaccine Targeting Cancer Neoantigens Enhances Efficacy of Anti-PD1 Therapy

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Background: Immune checkpoint inhibition (ICI) has revolutionized cancer therapy and significantly improved survival of patients across many different types of cancer. However, ICI is only effective in a subset of patients and most patients do not respond to ICI. Neoantigens are tumor specific antigens derived from somatic mutations within the cancer cells. They can also be derived from gene/RNA fusions and are recognized by the host immune system as foreign antigens. Several studies have shown that the success of ICI is linked to the number of neoantigens in the patient's tumor. Here, we demonstrate that a DNA vaccine targeting 40 neoantigens derived from MC38 mouse model of colon cancer synergizes with anti-PD1 antibody and improves the efficacy of anti-PD1 therapy. **Methods:** We performed whole exome sequencing on MC38 tumors to identify neoantigens. Based on the sequencing data, we identified 40 neoantigens based on predicted affinity to class I MHC binding. All 40 neoantigens were encoded into a single plasmid vector, each neoantigen separated by a furin cleavage site. Immune responses were measured in C57/Bl6 mice via IFN-γ ELISPOT assay and flow cytometry. Finally, we tested ability of MC38vax to control tumors *in vivo* and whether co-treatment with anti-PD1 antibody treatment improved tumor control. **Results:** Based on ELISPOT data, we observed that 11/40 neoantigens generated immune responses in mice. We also tested immune response to WT peptides and saw that the vaccine induced immune response was specifically against mutated peptides. Based on flow cytometry, we observed that the vaccine induced predominantly CD8+ T cell responses, although CD4+ T cell responses were also observed. In a

therapeutic tumor challenge, both anti-PD1 antibody and MC38vax as single treatment partially controlled the growth of MC38 tumors. However, co-treatment with both therapies demonstrated a 100% tumor control rate and significantly improved survival of tumor bearing mice. **Conclusions:** We have developed a DNA vaccine coding for 40 neoantigens that generated strong immune responses in mice. The vaccine significantly improved the efficacy of anti-PD1 antibody therapy and led to complete control of all tumors in an *in vivo* tumor challenge. Studies are ongoing to study whether the DNA vaccine can also improve the efficacy of anti-CTLA4 therapy.

1439 Immunosuppression Regimens in AAV Vector Gene Therapy Trials: A Longitudinal Review of a Cohort of Studies Conducted in the Last 5 Years

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The number of gene therapy clinical trials continue to increase, with over 500 Phase 1-3 trials being conducted in 2022. Untoward immune responses have been documented in recent literature for AAV vector-based therapies, including various inflammatory toxicities resulting from complement activation, cytopenias, and heterogeneous hepatotoxicities, to name a few. It is critical to understand, predict, and proactively manage these responses to delivery greater trial and investigational therapeutic safety to the patient community. Over the past 5 years, we have witnessed a clear change in how immunosuppression regimens are employed across a wide variety of clinical studies and indications. To understand how a subset of these regimens are evolving, a review of a series of greater than 20 protocols utilizing AAV vector-based gene therapies was performed. Reviewed protocols all had at least one patient enrolled within the last five years' and varied in clinical indication and route of gene therapy administration. Comparisons of the protocol version under which the first patient was randomized were made with the current protocol version to identify changes in immunosuppression regimens. Based on this review, there has been a trend to initiate immunosuppression regimens earlier in the clinical trial course and/or to alter dosing or titration schedules. Data will provide a summary of typical immunosuppression regimens, how the landscape has changed, and medications that are being included in more recent protocol versions to address untoward side effects. Additionally, examples that describe why regimen changes were made will be provided. This review shows that providing immunosuppression therapy at the time of gene therapy administration is one important way to support patient safety in these trials.

1440 Adeno-Associated Viruses (AAV) Displaying Single Domain Antibody Against AXL for Cancer Gene Therapy

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The use of adeno-associated viruses (AAV) as vectors for gene therapy has increased in recent years. Here, we incorporated an original single-domain antibody (sdAb) into the VP1 capsid of AAV serotype 2 (AAV2) to enable it to specifically bind to a receptor tyrosine kinase (Axl) widely expressed on many cancer cell lines, including SKOV-3 cells. The reduced size of sdAbs (~15 kDa) associated with high stability, specificity and their ability to bind with high affinity to their antigens make them promising alternatives to conventional antibodies and allow their incorporation into other proteins in viral vectors with minimal disruption of the native protein activity. In order to investigate their targeting efficacy, AAV with the modified capsid (AAV-sdAb) expressing GFP as a reporter were produced by transient transfection of HEK293SF-3F6 cells, purified by ultracentrifugation using iodixanol step-gradients and concentrated by Tangential-flow filtration using Hollow fiber membranes. To prove specificity for SKOV3 cells, plasmids required for AAV-sdAb production were mutated to inhibit the binding of AAV2 to its main receptor on the cell surface: heparan sulfate proteoglycan. Characterisation of this modified AAV-sdAb showed that the mutations led to a reduced tropism toward AAV2 natural receptor. More importantly, the viral vectors infected specifically the SKOV3 cells as a result of the inserted anti-Axl antibody. Competition experiments using heparin sulfate and the same anti-Axl sdAb that was incorporated into the AAV-sdAb, demonstrated that transduction of SKOV3 cells occurred through interaction with Axl. Therefore, this study proves that it is possible to re-target the AAV from its natural cell surface receptor to the receptor of interest by incorporating a sdAb into the capsid. Such strategy would be of great interest for cancer therapy by increasing the specificity of AAV for the delivery of toxic pay loads to cancer cells.

1441 Potent *In Vivo* CAR T Cell Generation and Durable Antitumor Activity in Preclinical Models Using VivoVec, a Surface-Engineered Lentiviral Vector Drug Product

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Autologous chimeric antigen receptor (CAR) T cell therapies have revolutionized the treatment of B cell malignancies, leading to long-term remission in a substantial number of patients. Despite the promising clinical efficacy of CAR T cells in hematologic malignancies, major limitations hinder their widespread application, including challenges for patient access, complex manufacturing, and high cost. To overcome these challenges, Umoja has developed VivoVec, a novel off-the-shelf surface-engineered lentiviral vector platform for *in vivo* CAR T cell generation. To achieve specific and efficient *in vivo* T cell transduction, VivoVec particles are surface-engineered with the cocal fusion glycoprotein and incorporate an anti-CD3 single chain variable fragment (scFv) and T cell costimulatory ligands on the particle surface to promote T cell binding, activation, and transduction. Here we demonstrate that CAR T cells generated with VivoVec particles exhibited a less-differentiated, central memory-like phenotype and enhanced CAR-antigen-specific polyfunctionality, including cytokine production, proliferation, and tumor cell killing *in vitro*. VivoVec particles have high selectivity and avidity for T cell binding. This enables efficient *in vivo* CAR T cell generation through multiple routes of administration, including direct injection or delivery via an extracorporeal gene delivery (ECGD) system. The ECGD system combines a standard leukapheresis collection followed by selective binding of VivoVec particles to T cells in a single closed-loop bedside procedure. The system has a binding chamber which enables consistent and controlled interaction between VivoVec particles and T cells. The binding properties afforded by the VivoVec surface engineering enable selective binding to T cells in the ECGD system in less than 1 hour, followed by an in-line wash to remove any unbound particles. The particle-bound T cells are then infused into the patient as part of the vein-to-vein bedside system. In a humanized NSG mouse model of B cell malignancy we observed that VivoVec particles generated substantial numbers of CAR T cells in the blood, resulting in potent and durable antitumor activity at low doses following direct *in vivo* administration or via the ECGD system. CAR T cell generation *in vivo* and CAR T cell mediated antitumor responses were enhanced by VivoVec particles surface engineered with T cell costimulatory molecules, as demonstrated by 20-50-fold higher numbers of CAR T cells in the circulation and greater antitumor responses at lower VivoVec doses compared to lentiviral particles lacking T cell costimulatory molecules. Our results demonstrate that incorporation of costimulatory molecules onto the surface of VivoVec particles promotes selective T cell binding, activation and transduction, resulting in the generation of potent polyfunctional CAR T cells *in vivo* that can rapidly clear primary a tumor and provide protection against tumor re-challenge. Overall, these data demonstrate that our surface-engineered VivoVec particles efficiently generate highly functional CAR T cells *in vivo*, following either direct injection or via the ECGD system . VivoVec particles have the potential to overcome many of the limitations associated with the current class of CAR T cell therapies.

1442 A Novel CRISPR-Cas9 Strategy to Target Dystrophin Mutations Downstream of Exon 44 in DMD Patient-Specific iPS Cells

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Duchenne Muscular Dystrophy (DMD), one of the most common forms of muscular dystrophy, is caused by mutations in the dystrophin gene. Although there are several approaches in the pipeline for clinical translation, there is still no effective treatment for DMD. An attractive approach is autologous cell transplantation utilizing human pluripotent stem (PS) cell-derived myogenic progenitors. Since rescue of full-length dystrophin is very challenging due to size constraints, here we leveraged the fact that a significant number of deletion mutations occur in the region between exons 45 and 55, known as the hotspot, and developed a gene knock-in approach to genetically correct any mutations downstream of exon 44. Our strategy consists of knocking-in the cDNA of exon 44 connected to exons 58-70. This introduces a miniature version of dystrophin protein (mini-DYS), which maintains the reading frame and the cysteine-rich domain that is essential for β-dystroglycan binding. We applied this approach to two DMD patient-specific iPS cell lines, GM25313 and cDMD012499-9, carrying mutations in exon 45 and 51, respectively, by integrating the short DMD cDNA via homology directed repair using CRISPR/Cas9-mediated double-strand break near the 5' end of exon 44. Knock-in was confirmed by PCR. Using conditional expression of PAX7, we differentiated gene corrected GM25313 and cDMD012499-9 iPS cell lines (2 clones each) and uncorrected counterparts into PAX7+ myogenic progenitors, and subsequently into to myotubes expressing myosin-heavy chain. Western blot and immunofluorescence staining confirmed mini-DYS expression at the protein level in the gene edited DMD iPS cell lines, but not in uncorrected counterparts. Spontaneous activity led to release of creatinine kinase (CK) into the medium, detected at lower levels in corrected DMD cultures. Transplantation of gene corrected DMD myogenic progenitors into NSG/mdx mice produced human donor-derived myofibers expressing both human-specific DYS and LAMIN A/C, suggesting *in vivo* rescue of dystrophin expression. These findings provide further proof-of-principle for the development of autologous iPS cell-based cell therapy for genetic disorders using programmable nucleases

1443 Enhancement of Newcastle Disease Virus AMHA1 Strain Oncolytic Activity through Inhibition of Heat Shock Protein 70 in Colorectal Cancer Cells. Clinical and Experimental Study

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The most frequent gastrointestinal cancer is colorectal cancer (CRC), which is a considerable global health concern. The colorectal cancer cells are stressed cells that express large quantities of intracellular and extracellular heat shock protein 70 (Hsp70) to correct distorted proteins, prevent cellular senescence, interfere with tumor immunity, enhance angiogenesis, and help metastatic formation by increasing resistance to treatment. They also secrete Hsp70 outside the cell, affecting immune cells directly and increasing cytokines production. Therefore, Hsp70 inhibition as a targeted therapy becomes a necessity. The current work aimed to Investigate the increased expression levels of Hsp70 in colorectal cancer patients' serum and cancer tissues and its possible targeting for cancer therapy. We targeted colorectal cancer cells by combining Hsp70 inhibitor KNK437 with NDV AMHA1 Iraqi strain to prevent cancer cells from reducing the stress initiated by NDV. **Methods**: The clinical study was conducted on 94 individuals divided into two groups. Group I: 54 patients diagnosed with colorectal cancer, and Group II: 40 patients diagnosed with different colorectal diseases with no malignancy. We collected serum and tissue to investigate the Hsp70 level of expression. Moreover, we checked IL-1 and IL-10 cytokine levels in serum samples of CRC patients to highlight this pathway's importance in patients' immune status, which may affect CRC progression that has a strong relationship with hsp70 pathways. In vitro study utilized a colorectal cancer cell line, HRT-18G as a model to study the inhibition of Hsp70 and the cytotoxicity of Newcastle disease virus (NDV) as an oncolytic virus. Hsp70 expression was checked in the HRT-18G, which was found to be elevated. **Results**; An immunohistochemical (IHC) study showed that Hsp70 was positively detected in all colorectal cancer tissue samples with significant ($p < 0.0001$) intracellular expression compared with non-malignant, healthy tissue samples. Extracellularly, the serum levels of Hsp70 and IL-1, and IL-10 were significantly ($p < 0.0001$) elevated in CRC samples compared with the control. To translate the clinical finding into experimental targeted therapy as proof of concept, we targeted Hsp70 using KNK437, which induces significant inhibition of Hsp70 in CRC cells *in vitro*. Cancer cells exposed to NDV showed a substantial killing effect compared to untreated cells. The combination therapy of NDV and KNK437 (Hsp70 inhibitor) showed synergism in reducing the viability of CRC cells with a neglectable effect on normal cells when analyzed using the Chua-Talalay equation. Hsp70 expression levels in CRC cells were investigated using qPCR, IHC, and ELISA after monotherapy and combination therapy. The expression of Hsp70 was suppressed by KNK437 and NDV-KNK437 combination therapy but elevated when the CRC cells were infected with NDV. These findings suggest that combination therapy kills through increasing stress on cancer cells by suppressing Hsp70, resulting in higher cytotoxicity against cancer cells. Acridine orange/propidium iodide double staining confirmed the ability of combination therapy to induce apoptosis in colorectal cancer cells at a higher level than applying individual therapies. In conclusion, oncolytic NDV AMHA1-KNK437 combination therapy enhanced the antitumor activity against colorectal cancer cells and demonstrated strong synergy that suggests a novel anti-colorectal cancer treatment that can be applied clinically.

1444 Conditional Regulation of rAAV-Delivered Hepatic Therapeutics by Iron and miRNA-210-3p

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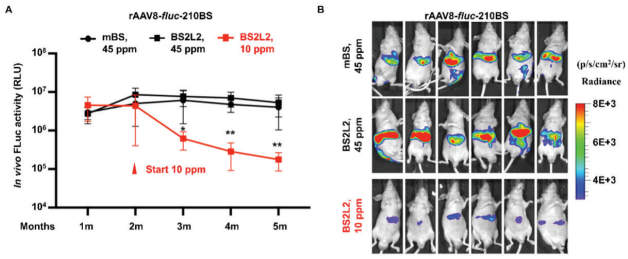
Liver gene therapy based on recombinant adeno-associated virus (rAAV) vector has achieved great success, but the regulation of transgene expression has remained a problem. It has been reported that incorporating tissue-specific miRNA binding sites into rAAV expression cassettes could de-target transgenes from specific cell types. Iron, one of the most extensively studied nutrients, is recycled and stored mainly in the liver with a wide normal range. To date, the crosstalk between miRNA and iron homeostasis, especially within the normal range, is not clear. In the current study, we aimed to take advantage of a hepatic miRNA that is specifically regulated by iron at physiological levels, in order to achieve control of rAAV-mediated transgene expression in the liver.Here, we reported that the expression of miR-210-3p, whose baseline level is low in the liver, was dramatically upregulated by diet iron in both normal and immune-deficient mice. Though miR-210-3p is not liver-specific, we observed that miR-210-3p was up-regulated most significantly in the mouse liver (17.6-fold).We further explored the underlying molecular mechanism. We found that the methylation status of the promoter region of miR-210-3p (m210p) was significantly changed after iron chelator treatment. When m210p was cloned to a GLuc plasmid and transfected into Hepa1-6 cells, *gluc* expression could be enhanced by iron chelator. When we mutated the hypoxia-inducible transcription factors (HIF) 1α binding sites in the m210p, iron chelator could no longer enhance the *gluc* expression. Given that DNA methylation is central to binding of HIF on promoter regions and that HIF pathway is well known to control iron-related gene expression, it was evident that HIF1α binding sites in the miR-210-3p promoter are essential for iron-mediated upregulation of miR-210-3p.Finally, we showed that the hepatic transgene expression from miR-210-3p binding site-incorporated rAAV vectors could be significantly regulated by iron-chelator *in vitro* and diet iron *in vivo* (Fig.1). Most importantly, we showed that the regulation could be applied to not only reporter genes, but also a CRISPR-effector gene.In summary, we identified miR-210-3p that significantly response to iron status in the liver, revealed its molecular mechanism, and exploited it to achieve control of rAAV-mediated transgene expression in the liver. Considering the simple oral route and the comprehensive safety profile of diet iron, our studies provide a unique opportunity to improve the safety and efficacy of liver-targeted gene therapies.



1445 Development of High Yield Plasmid and mRNA Manufacturing Process in Scalable Level

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In recent years, mRNA technology has been widely used in treating infectious diseases, cancer, and rare diseases due to its remarkable platform technology advantages. The safety and efficacy of mRNA therapeutics have been demonstrated in COVID-19 vaccines. Process development is a critical step during the development of mRNA therapeutics, which includes plasmid and template preparation, in vitro transcription (IVT) reaction and mRNA purification. In this study, our team has developed a process development platform for plasmid and mRNA manufacturing with optimized critical process parameters (CPP) of the main steps. By optimizing the concentration of NaOH in alkali lysis buffer, the yield of plasmid was increased by 36%. By optimizing the concentration of calcium chloride added into neutralized lysate, the residual host cell RNA (HCR) levels in neutralized lysate were significantly reduced. After UF/DF, crude plasmid samples were purified by gel filtration chromatography, the residual host cell protein (HCP) and HCR were reduced to 0.1 μg/mg plasmid by ELISA and qPCR analysis. After further purification by hydrophobic chromatography, the host cell DNA (HCD) residue was controlled within 2 μg/mg plasmid. The open-loop plasmid and linearized plasmid impurity was controlled less than 2% with the supercoiled plasmid over 95%. In linearization stage, by optimization of plasmid concentration, enzyme concentration, reaction time and other parameters, the purity of the linearized plasmid was more than 97% by AEX-HPLC analysis. AGE analysis of linearized plasmid showed a clear single band. In IVT reaction processing, the optimization lies in the source and concentration of T7 polymerase, the concentration of the linearized template, NTP material, cap analogues, and the reaction time. The mRNA concentration and purity reached 12 mg/mL and more than 90% when IVT reaction is processed under optimized conditions. In the final product, the dsDNA content was found less than 0.5% and the purity of mRNA more than 95% after Oligo dT affinity chromatography purification. The optimized IVT and purification process of have been verified at the scale of 3,000 mg mRNA drug substance. In summary, OBiO's process development platform demonstrated high yield, high purity mRNA manufacturing capabilities by optimizing CPPs mentioned above.

1446 Development of *In Vitro* Neuronal Cytotoxicity Models for Neurodegenerative Disease Gene Therapy R&D

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Globally, neurological disorders such as Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), and Frontal Temporal Dementia (FTD) are the second leading cause of death, with millions of new diagnoses each year. ALS alone affects 1 in 50,000 people per year worldwide, calling for increased demand in efficient therapies. Cytoplasmic mislocalization and the subsequent accumulation and aggregation of transactive response DNA-binding protein 43 kDa (TDP-43) is a hallmark of ALS and, in most cases, represents a reliable post-mortem diagnostic marker. Multiple studies have demonstrated that overexpression of TDP-43 in animal models results in neurodegeneration and can be used as a disease model for ALS and other neurological disorders involving TDP-43. While valuable, *in vivo* TDP-43 disease models are hindered by their low throughput capabilities. Here, we propose two cellular models of TDP-43 induced cytotoxicity mediated by adeno-associated virus (AAV) transduction. First, primary cortical mouse neurons were transduced with AAV containing TDP-43 and cytotoxicity was tracked over time. Dose-dependent cytotoxicity, as well as changes in morphology, were observed in the TDP-43-transduced neurons. The same experiment was conducted using ReNcells, an immortalized human neural progenitor cell line. Like for primary cortical mouse neurons, treating ReNcells with AAV-TDP-43 resulted in dose-dependent cytotoxicity determined by LDH activity and AO/PI staining. Our data demonstrate that these *in vitro* models have the potential to be used in high throughput screens and functional potency assays for neuroprotective gene therapies development.

1447 Non-Evolved dCas9 Epigenetic Editors Targeting NGG PAM Sites Are Required for Knockdown of *HTT*

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Huntington's Disease (HD) is a fatal, autosomal dominant neurodegenerative disorder caused by a trinucleotide repeat in exon 1 of the Huntingtin (*HTT*) gene. This expansion leads to protein misfolding that causes widespread cellular dysfunction and ultimately leads to cell death. There are currently few treatments and no cures, with death occurring 10-15 years after disease onset. The advent of CRISPR-dCas9 technologies allows for the targeting of the causative gene and subsequent downregulation via fused effector domains that elicit an effect at the epigenetic level to induce heterochromatin and block transcription. Allele specificity is important in HD, as HTT has essential functions within the cell and total knockdown could be deleterious. HD has large haplotype blocks that allow for allele-specific targeting based upon the presence of heterozygous single nucleotide

polymorphisms (SNPs). These SNPs are in genomic regions devoid of NGG PAM sites, a requirement for SpdCas9 binding. To address this bottleneck, we conducted a screen of multiple dCas9 variants fused to KRAB and DNMT3A/L with increasingly expanded PAM targeting to assess the ability to downregulate *HTT* and provide allele specificity. Surprisingly, only SpdCas9 was able to significantly knockdown *HTT*, while expanded PAM site variants dxCas9 and dCas9-VQR were unable to reduce *HTT* expression. Locus occupancy analysis by ChIP-qPCR was performed to identify differential binding enrichment of dCas9 variants. We further investigated these effects by quantifying epigenetic changes at the promoter through bisulfite sequencing for DNA methylation and ChIP-qPCR for H3K9me3. We then assessed knockdown in neuronal stem cells from an HD patient and healthy controls. We measured knockdown efficiency in our healthy controls and HD patient cells by qPCR. In addition, we have shown the ability to package this dCas9 system into a viral-like particles (VLP) and show subsequent delivery and knockdown of *HTT in vitro*. Such VLP strategies have shown growing promise through co-opting the viral packaging system to direct packaging of ribonucleoprotein complexes. In conclusion, this platform can be modified for application in multiple neurologic diseases that will allow for the target and treatment of previously untreatable disorders that were limited by a dominant negative etiology.

1448 Engineering an Automated Platform for High-Throughput Production of Plasmid DNA for Cell and Gene Therapy

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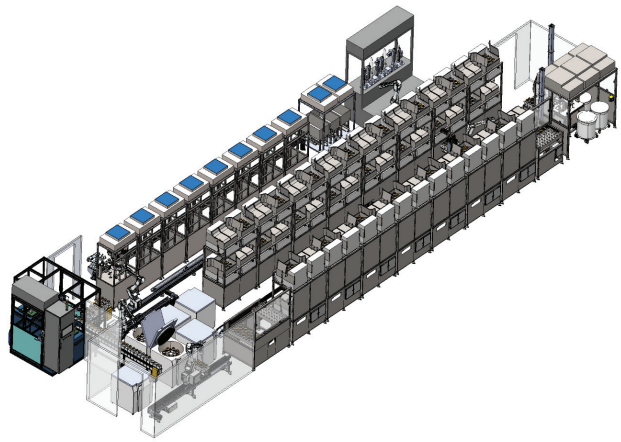
Genscript, Piscataway, NJ

The global demand for plasmid DNA has increased rapidly since the advent of cell and gene therapies (CGT) followed by hyper-growth during the outbreak of the COVID-19 pandemic. These cutting-edge therapies come in many forms but all of them rely on plasmid DNA at some point either in their manufacture or mechanism of action. Unfortunately, scaling up manufacturing capacity for plasmid DNA is a difficult operation and it has become a bottleneck for the CGT industry. Most companies outsource plasmid production to partners such as contract development and manufacturing companies (CDMOs) who are specialists in the arena and have in-house manufacturing platforms. CDMOs can no longer meet the demand for high quality plasmid DNA due to substantial backlogs and the lack of automated solutions for plasmid manufacturing. This can significantly impede R&D efforts and increase time-to-market delaying access for patients to these potentially curative therapies. Here we present GenScript's new high-throughput plasmid production platform designed to produce up to 1000 ultra-low endotoxin plasmids at the mg scale per day. This is an integrated platform bringing the best of industrial automation to a biological manufacturing process. The platform consists of six robot operated modules encompassing inoculation, incubation, lysis, chromatography and solvent extraction. It can operate continuously in a 24 hour cycle with in-process cleaning to prevent cross-contamination (Figure 1). All sample handling will be under HEPA-filtered enclosures to prevent contamination. We also present a new paradigm for bacterial culture and lysis in single-use bottles instead of flasks or fermenters. We have demonstrated that we can obtain yields of >0.7mg for both low and high-copy plasmids with endotoxin levels < 0.05EU/μg and supercoiled content >80% (Figure 2). Our platform can produce transfection grade plasmid libraries for AAV capsid libraries, antibody libraries, CRISPR libraries and *in vitro* transcription (IVT) templates for mRNA production for the CGT industry. GenScript will accelerate the commercialization of new CGT products by providing high quality plasmids with a two day turnaround time.



1449 The Therapeutic Effects of Antisense Oligonucleotides in iPSC-Derived Neurons of Patients with LBSL

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Background: Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) is an ultra-rare inherited mitochondrial disorder characterized by progressive spastic gait, ataxia, and posterior spinal cord involvement. LBSL is caused by mutations in *DARS2*, which disrupt the mitochondrial aspartyl-tRNA Synthetase. Compound heterozygote mutations consisting of one mutation in intron 2, affecting splicing of exon 3, and one missense mutation have been found in over 90% of the identified cases with LBSL. It is postulated that restoring *DARS2* mRNA expression by increasing exon 3 inclusion may improve protein function and result in a positive clinical outcome. We therefore aimed to design an antisense oligonucleotide (ASO) targeting *DARS2* mRNA in an attempt to enhance exon 3 inclusion and restore gene expression (Figure 1). **Methods:** Fifteen ASOs were designed to target possible intronic splicing silencers upstream of exon 3. The ASOs were 20 base pairs in length. All nucleotides included 2′-O-methoxyethyl (2′-O-MOE) and phosphorothioate (PS) modifications to enhance ASO stability and prevent RNase-H activity. Induced pluripotent stem cells (iPSCs) were derived from blood cells of three LBSL patients and one healthy control. iPSCs were transfected by ASOs to find the most effective ASO in increasing exon 3 inclusion by RT-qPCR. The ASO with the highest inclusion efficacy was tested in two doses (50nM and 100nM) on iPSC differentiated neural progenitor cells (NPCs). In addition to RT-qPCR for inclusion/exclusion of exon 3, lactate was assessed (Lactate Reagent Set, Pointe Scientific) on the cultured media of iPSC differentiated motor neurons to evaluate mitochondrial function. **Results:** The ASO targeting 180 to 200 nucleotides upstream of *DARS2* exon 3 revealed the

highest elevation in exon 3 inclusion, compared to the other designed ASOs and untreated iPSCs. RT-qPCR of NPCs transfected by this ASO showed increased levels of *DARS2* mRNA including exon 3 and reduced levels of *DARS2* mRNA excluding exon 3 in three LBSL cell lines in a dose dependent manner. Furthermore, 30-day motor neurons treated with ASO for one week demonstrated lower levels of lactate, compared to the baseline lactate levels of LBSL neurons. Reductions in lactate levels were observed in a dose dependent manner. **Conclusions:** ASO therapy shows promise to restore gene expression in iPSC derived NPCs of patients with LBSL. The role of ASO in modulating lactate levels in LBSL neurons suggests improvements in mitochondrial function.

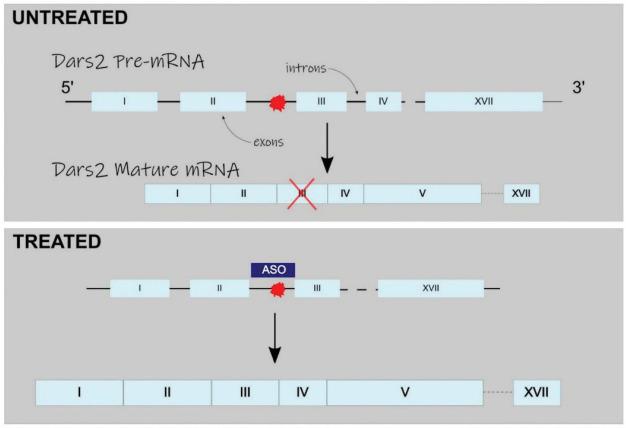

Figure 1. Most patients with LBSL have reduced exon 3 expression within *DARS2* mRNA due to a splice site mutation in intron 2. Antisense oligonucleotides (ASOs) targeting intronic splicing silencers upstream of exon 3 may restore gene expression.

1450 3D Bioprinted Liver Tissues Rescue a Murine Model of CCl_4- Induced Acute Liver Failure

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Aspect Biosystems, Vancouver, BC, Canada

Background: The standard of care treatment for Acute Liver Failure (ALF) and Acute Chronic Liver Failure (ACLF) is liver transplantation. However, due to the limited supply of suitable donor organs, a high proportion of patients with liver failure die while waiting for a liver transplant. The transplantation of isolated primary hepatocytes is a promising treatment for pediatric patients with ALF; however, implementation and scaling of this treatment remains a challenge. In this study, we demonstrate that xenogeneic 3D bioprinted liver tissue therapeutics, containing primary human hepatocytes (PHHs), and mesenchymal stromal cells (MSCs), are capable of rescuing immunocompetent mice from Carbon tetrachloride (CCl_4)-induced ALF when implanted into either the IP or SubQ space, without the need for immunosuppression. **Methods:** Prior to printing, PHHs and MSCs were formed into spheroids and then suspended in an alginate-based biomaterial. Liver tissues containing 5 million hepatocytes and 5 million MSCs were manufactured using Aspect Biosystems' microfluidic 3D bioprinter. For the study, immunocompetent C57BL/6 mice were injected with titrated CCl_4 to induce liver failure. After 1 day, the mice were implanted with 2 liver tissues into either the IP or SubQ space. Control mice treated with CCl_4 were implanted with cell-free bioprinted tissues. For analysis, blood was collected at -7, 1, and 7 days post-surgery to measure endogenous liver damage using alanine transaminase (ALT) levels, and implanted liver tissue function via human albumin levels. Animals were euthanized at the end of the experiment and the liver was collected for histological examination. **Result:** Implantation of bioprinted liver tissues containing human cells significantly improved survival in mice with CCl_4-induced liver failure. CCl_4-treated mice that received liver tissues into the IP space had a survival rate of 78% vs 47% for control CCl_4-treated mice. Mice that received liver tissues in the SubQ space demonstrated equivalent efficacy, with a survival rate of 85% vs 50% for control mice. This experiment was repeated with hepatocytes from an alternate donor. Upon replication, the survival rate for treated mice was 50% vs 13% for cell-free implanted mice. Across all three experiments, the survival rate for CCl_4-treated mice was 68% for animals that received PHH/MSC tissues vs 35% for mice receiving cell-free control tissues. The p-value was 0.003. ALT levels peaked on day 1 post-surgery at over 1000 U/L. Liver histology of mice that succumbed to liver failure before day 7 showed extensive liver necrosis. Treated and untreated mice surviving until the end of the experiment showed significant evidence of liver regeneration and normal liver parenchyma restoration. **Conclusion:** Our data demonstrates that 3D bioprinted liver tissues implanted IP or SubQ significantly reduced mortality of immunocompetent mice from CCl_4-induced ALF. As such, 3D bioprinted tissue therapeutics have the potential to act as a bridge to recovery or transplantation for individuals with ALF or ACLF. Our results also suggest that 3D bioprinted hepatocytes could be applicable in the treatment of other chronic metabolic liver diseases caused by a functional deficiency in hepatocytes. Our next steps are scaling the therapeutic to larger cell doses to examine efficacy in a rat model of ALF and a mouse model of chronic metabolic liver disease.

1451 Characterization of rAAV8 Vectors from Six Chinese CRO Companies

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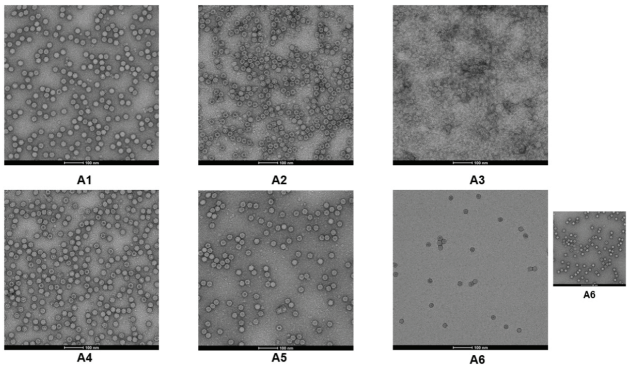
Recombinant adeno-associated virus (rAAV) has drawn substantial attention as a highly promising vector for human gene therapy worldwide. rAAV vectors currently become an increasingly popular delivery tool in preclinical and clinical applications. However, it is evident that the current rAAV vector production especially in China will require improvements in quality before it can be used in clinical since its systematic production process did not begin until 2016. In this report, we characterized the quality of rAAV8 vectors produced by six Chinese CRO companies, called A1, A2, A3, A4, A5 and A6 respectively. First, genome titer, capsid titer, full capsid ratio and rAAV serotype identity were simultaneously determined through traditional methods and Stunner/Uncle machine. Although the quality data of six rAAV8 vectors under the detection of two methods were slightly different, the main trend was consistent, except for A4, in which there was too much iodixanol impacting machine detection.In terms of genome titer, A1 and A4 were the highest, A3 and A6 were the lowest,

while the A2 genome titer obtained from machine was overestimated due to excess cell-free DNA. Next, in the detection of capsid titer, A1 and A2 were the first level, and A6 was the lowest, the largest difference spanned 10 times. As for the full capsid ratio detection, the results gained from qPCR combined with Elisa showed that A6 was the highest, while A2 and A3 were the lowest. Due to the data processing method, the full capsid ratio determined by machine was meaningless. The transmission electron microscope (TEM) results showed that A1 was the highest, followed by the A4, A5 and A6 (Fig. 1). Owing to too many impurities, A3 could not be analyzed, which was also reflected in the follow-up staining. A3 was too impure to be detected for rAAV serotype identity, while the other samples all identified as the same serotype. Western blot showed that all the rAAV capsids were intact, but A3 was too impure to be detected by coomassie blue staining and silver staining. The concentration of A6 was so low that the band was too faint.Furthermore, transducing unit was analyzed, demonstrating that A1 was the highest, and A6 was the lowest. The value of transducing unit divided by genomic titer represents rAAV potency, where A1 to A4 were similar, while A5 and A6 were lower than the first four about 10 times. Finally, we detected the neutralizing antibody (Nab) titer activated by six rAAV vectors. Surprisingly, the Nab titer activated by A1 was comparable to that of A3. The other three except for A6 were all low, since A6 was insufficient to inject mice due to its low concentration.Taken together, A1 was of the highest quality, but still resulted in higher Nab titer. We are the first to systematically compare the quality of purified rAAV vectors produced by Chinese CRO companies, presenting the fact that they each have own strengths and weaknesses, providing convenience for the better application of rAAV vectors in the Chinese scientific research community.



1452 Biodistribution of LV-TSTA Transduced Rat Adipose Derived Stem Cells Used for "*Ex Vivo*" Regional Gene Therapy for Bone Repair

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INTRODUCTION: Massive bone loss remains a difficult clinical entity. As ex vivo and in vivo gene therapy are explored as potential treatment methods for critical sized bone defects, an assessment of their safety becomes important. Previously, we investigated the safety of lentiviral transduction of bone marrow-derived mesenchymal stem cells (BMSCs) to overexpress bone morphogenetic protein 2 (BMP-2), and found that lentiviral copy numbers declined at the defect site and were not consistently found in various internal organs at subsequent points. Subsequently, we observed greater growth potential and BMP-2 production for adipose derived stem cells (ASC) compared to BMSCs. In this study, we sought to characterize the biodistribution of viral particles at the defect site and various internal organs after implantation of ASCs transduced via lentivirus to overexpress BMP-2. METHODS: Under IRB approval, adipose cells were harvested from the infrapatellar fat pad of donors. ASCs were then isolated and cultured. Two-step transcriptional activation (TSTA) was utilized to create vectors containing bone morphogenic protein (LV-TSTA-BMP-2) and green fluorescence protein (LV-TSTA-GFP). 5 experimental groups were created, including: High-dose (HD) LV-BMP-2, standard dose (SD) LV-BMP-2, HD-LV-GFP, SD-LV-GFP and non-transduced. Utilizing a critical sized femoral defect model, cells were implanted into 12-week old athymic nude rats. Timepoints examined included 4, 14, 21, 56 and 84 days. Outcomes examined included viral copy number and histologic examination of the implant site and internal organs, liver function tests, and integration site analysis. Organ histopathologic analysis was performed by a clinical pathologist. Statistical analysis was performed using STATA17 with Wilcoxon signed-rank test or ANOVA for continuous variables and a chi-squared analysis for categorical variables. Statistical significance was set at $p<0.05$. RESULTS SECTION: Viral copy number at the defect site remained present through three weeks in both the high dose LV-TSTA-BMP-2 and standard-dose LV-TSTA-BMP-2 groups (Figure 1). Across the time points, there was a significant decrease in viral copy number at the fracture site in the cohorts ($p<0.0001$). On histologic examination, none of the samples across all time points and organs examined were noted to be abnormal. Furthermore, there was no significant number of viral copies or human cells detected in the various organs (brain, lung, heart, GI tract, liver, spleen, kidney, testis, bone marrow and blood) between 2 and 12 weeks for either the high dose or standard dose groups. Integration site analysis performed for 3 samples did not yield significant clonal abnormalities or integration sites near genes of interest. DISCUSSION/CONCLUSION: In this study, we demonstrate that LV-TSTA-BMP-2 transduced cells remain at the femoral defect site for 3 weeks before significantly diminishing in numbers. Our histopathologic data suggests that this method appears to be safe with no instances of abnormal histology noted. Furthermore, there was no significant detection of viral copies outside of the femoral defect site after 4 days in the high-dose and standard-dose LV-TSTA-BMP-2 cohorts.. Together, these data suggest that transduced ASCs remain a viable option for ex-vivo gene therapy.

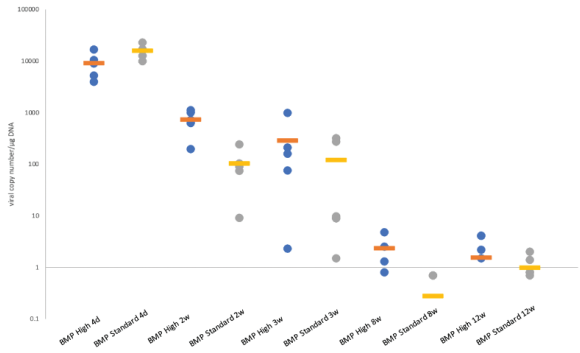

Fig 1. Viral copies per µg DNA detected in defect site by experimental group and time point.

1453 Development of an *Ex Vivo* Gene Engineered B Cell Medicine Platform with Precision, Modularity, and Broad Therapeutic Utility

Hanlan Liu[1], Tim Mullen[1], Swati Singh[2], Jasmine Edelstein[1], Carolline Bullock[1], Sean Keegan[2], Sarah Leach[1], Troy Patterson[1], Kate Molloy[1], Amy Lundberg[1], Michael Leiken[2], Ishara Datta[1], Wayne Bainter[1], Claudia Fiorini[2], Sean Arlauckas[2], Anja F. Hohmann[2], Adam Lazorchak[2], Richard A. Morgan[2]

[1]Be Bio, Cambridge, MA,[2]Be BioPharma, Cambridge, MA

Ex vivo gene and cell therapies have produced transformative medicines to treat previously intractable diseases. Still, there are barriers to the broad adoption of these efficacious therapies as current platforms lack high biosynthetic capacity, durability, and cannot be administered as an off-the-shelf medicine without conditioning. Terminally differentiated human plasma cells derived from genetically engineered B cells (termed B Cell Medicines, BeCMs), offer natural longevity (mean half-life of 17 years), capacity for high levels of protein secretion (up to 10,000 Ig molecules/cell/sec), and naturally allogenic properties, which make them an attractive platform for the sustained supply of biologics, where continuous dosing is required to achieve therapeutic benefit. Herein we describe a modular and scalable platform for the generation of BeCMs capable of stable expression and continuous secretion of biologically active therapeutic cargos. BeCMs are produced via CRISPR-based HDR-mediated transgene insertion followed by ex vivo differentiation into long-lived plasma cells. A BeCM prototype engineered to express firefly luciferase was produced and differentiated into antibody secreting cells (ASC) with a phenotype of >90% CD27+CD38+ cells. The BeCM prototype was injected via IV into immunodeficient mice, with rapid (< 3 days) bone-marrow-homing and durable engraftment (>100 days) . To illustrate the modularity of the BeCM platform we engineered B cells to produce the lysosomal storage disease (LSD) enzyme acid sphingomyelinase (ASM), clotting factor IX (FIX), and an anti-CD19/CD3 bispecific T cell engager (BiTE). ASM deficiency caused by lack of SMPD1 gene expression leads to Niemann-Pick Disease (NPD), an autosomal recessive LSD that causes sphingomyelin accumulation in organs leading to organ failure and neurodegenerative damage. BeCMs harboring an optimized SMPD1 expression cassette inserted into the CCR5 safe harbor locus was demonstrated to stably secrete ASM. HAP1 knockout cells exposed to supernatant from SMPD1-engineered BeCMs showed restored ASM activity, and reduced accumulation of pathological sphingomyelin as demonstrated by liquid chromatography-mass spectrometry. Hemophilia B is an X-linked coagulation disorder caused by deficiency of functional FIX protein (FIX). Insertion of a FIX transgene construct at the CCR5 locus and subsequent differentiation into ASCs, resulted in FIX production with biological activity as measured by a chromogenic enzyme activity assay and activated partial thromboplastin time assay. FIX-expressing BeCM were transferred into immunodeficient mice, with FIX production demonstrated >12 weeks in vivo. As a third example, we engineered B cells to produce an anti-CD19/CD3 BiTE mimicking the drug Blincyto®, an approved continually-dosed therapy for relapsed/refractory Acute Lymphoblastic Leukemia (ALL). Supernatant from BiTE-producing BeCMs directed primary human T cells to kill Raji tumor cells (CD19+), demonstrating potent in vitro anti-tumor activity. In summary, we demonstrated the ability to transform the power of B cells into a platform for the advancement of B cell-based medicines. Successful production of highly divergent biologics such as LSD enzymes, serum proteases, and anti-tumor bispecific engagers highlight the therapeutic versatility and modularity of our BeCM platform. BeCMs capable of expressing one or more therapeutically relevant transgenes have the potential for broad and meaningful therapeutic utility in rare diseases, cancer, and beyond.

1454 Human Anti-SARS-CoV-2 Tetravalent Bispecific Antibody Broadly Neutralizes Major Variants of Concern Including Omicron Sub-Variants

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Nearly 80 variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been recorded, many of which, such as the heavily mutated Omicron variants, have rendered the existing monoclonal neutralizing antibody (nAb) treatments futile. Therefore, we developed a rapid platform to vectorize and screen anti-SARS-CoV-2 nAb codes deciphered from convalescent patient-derived memory B-cells. To achieve this, we vectorized the spike (SARS-CoV-2 envelope glycoprotein)-specific antibody codes into 3rd generation self-inactivating lentiviral vectors and produced recombinant human antibodies in HEK-293-T cells. To screen nAbs for their neutralizing capacities against viral escape, we incorporated all receptor binding domain (RBD) mutations from the recorded variants or variants

of concern (VOCs), namely Wuhan, Alpha, Beta, Gamma, Kappa, Delta+ (AY.2), IHU Méditerranée and Omicron BA.1, BA.2, BA.4/5, and engineered their replication-incompetent pseudotyped vectors (pseudoviruses) using known retroviral vectors. Using a flow-cytometric assay to detect pseudoviral infection, we screened over 90 monoclonal nAbs and identified three (C9, A7 and A9) that neutralize the Wuhan strain pseudovirus at IC_{50} concentrations of 6, 9 and 22 ng/mL respectively, similar to the approved therapeutic antibodies. While all three monoclonal nAb candidates were effective (≈70-100% neutralization) against Alpha, Beta, Gamma, Kappa and Delta+ variants, they displayed lower efficacy (≈0-50%) to neutralize the Omicron BA.1 and IHU Méditerranée variants in a monotherapeutic setting. Existing nAb cocktail therapies (e.g. REGN-COV2 and Evusheld) have demonstrated the potential of two monoclonal nAbs to circumvent viral escape. Based on this evidence, we designed and produced an innovative tetravalent bispecific candidate (A7A9 TVB nAb) against immune evasion of SARS-CoV-2 variants. A7A9 TVB nAb, which consists of four antigen-binding arms with specificity towards two distinct epitopes on the RBD, neutralized all tested VOCs better than its parental clones (except for Omicron BA.4/5, which has often been reported to evade neutralization by antibodies in other studies). Moreover, on differentiated primary nasal epithelium (DPNE, as an air-liquid interface culture) infected with authentic (full-length wild-type virus) Omicron BA.1, pre-incubation of the virus with A7A9 TVB nAb showed significantly reduced viral titers 24 hours post-infection as measured via a plaque assay on Vero E6 cells. Furthermore, stable A7A9 TVB nAb-expressing DPNE against authentic Omicron BA.1 showed a consistent decline in viral load, leading to viral clearance five days post-infection. These results strongly corroborate the clinical relevance of A7A9 TVB nAb. Additionally, to address the aspects of stability and specificity, we characterized all antibodies for their size and integrity using western blot analysis and validated their unique binding specificities through cell-based binding assays using stable cell lines expressing spike domains (S1, S2, NTD, RBD, NTD + RBD) as surface antigens. In summary, we have generated a new vectorization and screening platform for development of antibodies with modular designs that can be expanded to other infectious pathogens with urgent medical needs and beyond.

1455 Standardized Transfection: The Key to Robust and Scalable rAAV Production Processes

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To date, the majority of production processes for recombinant adeno-associated viral (rAAV) vectors are based on the transient transfection of HEK293-derived cell lines with plasmids containing the genetic information for essential viral components. The key challenges of these production processes are characterized by poor process robustness and scalability, particularly arising from the high complexity and variability of the transfection unit operation. This makes rAAV production processes very expensive resulting in a limited availability of gene therapies for patients. To address these challenges and increase robustness of the transfection unit operation, we started to identify and understand the critical material quality attributes of our raw materials. This forms the basis for an adequate raw material control strategy and ultimately helped us to reduce transfection variability. Going further, we developed standardized protocols for the preparation of the transfection complexes and the addition of those at various scales. Using this approach, we were able to establish a robust suspension process that is scalable from benchtop up to 500-liter scale. To further improve our understanding of the impact of raw material attributes and transfection parameters on process performance and quality attributes of the rAAV, we are evaluating methods for transfection complex characterization. We are convinced that an in-depth understanding of the transfection complex formation will accelerate the path towards more robust and scalable rAAV production processes.

1456 Next-Generation DNA-Based Delivery of Therapeutic Proteins Using MYO Technology: Preclinical Results on Filgrastim and Neutropenia

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Therapeutic proteins, as a class of drugs, have seen a substantial increase in both clinical development and use. Any protein-based drug belongs to this class, including antibodies, hormones, engineered protein scaffolds, enzymes, interferons, anticoagulants, growth factors, and many others. Despite the broad applicability of these drugs, their more widespread use is often limited by high manufacturing costs and hurdles to delivery. Many therapeutic proteins are administered via time-consuming intravenous infusions and others, regardless of administration route, need to be dosed frequently, sometimes even daily. Another drawback of many biologics is the requirement for maintenance in a low temperature environment prior to use. Such cold-chain dependencies can significantly hinder the ability to get therapeutics to large numbers of people, a problem that is amplified in resource poor settings. MYO Technology™, a DNA-based platform for the delivery of therapeutic proteins, was developed to overcome these barriers. The MYO Technology platform consists of therapeutic-encoding plasmid DNA (pDNA) and a proprietary medical device for the intramuscular injection of pDNA, followed by the delivery of very short electrical pulses to the muscle tissue surrounding the injection site. These pulses promote the in vivo electroporation of muscle cells and uptake of pDNA, leading to production and secretion of the therapeutic protein, and ultimately uptake into peripheral circulation. Unlike many of these proteins, pDNA is simple to manufacture, and the process is less specialized. Additionally, pDNA is very stable and lacks most cold chain requirements. Moreover, administration using MYO Technology takes just a few minutes, and the serum level of a therapeutic protein can potentially be maintained for many months. This contrasts with many therapeutic proteins that require frequent dosing when administered by standard methods.One example of such a therapeutic protein is filgrastim, also known as granulocyte-stimulating

factor. It is a bone marrow stimulant, indicated for the treatment of neutropenia. Filgrastim is very potent and well tolerated by patients, but is characterized by a half-life of only ~3.5 hours and typically requires daily administration by subcutaneous injections. Severe chronic neutropenia patients may have a life-long dependency on filgrastim, and this requirement for daily drug injections not only negatively affects quality of life, but also can impact adherence to therapy.Here, we present our preclinical studies on delivery of filgrastim with MYO Technology. Animal proof-of-concept studies demonstrate that in vivo-produced filgrastim is functional and efficacious in improving neutrophil count, both in healthy animals and in mouse models of neutropenia. Expression of MYO Technology-delivered filgrastim is pDNA-dose dependent, and remains stable for several months or more. Importantly, studies in larger animal models demonstrate the ability to scale up from smaller species.Through significant improvements in administration frequency, MYO technology has the potential to dramatically improve quality of life and therapy adherence among severe chronic neutropenia patients. Furthermore, MYO Technology may present the same advantages to other therapeutic proteins with short half-lives.

1457 A Scalable, Automated Platform for Developing the Next Generation of CAR-T Therapies

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Identifying the genetic determinants of CAR-T function is essential to engineering the next generation of cell therapies. Current methods for optimizing CAR-T employ genetic screening to identify genes that enhance CAR-T safety and efficacy. Functional profiling of such edited T cells is best accomplished using an arrayed—rather than pooled—screening approach, as arrayed screening provides superior genome coverage and allows phenotypic profiling of individual gene edits. The accessibility of arrayed screens is limited by high costs (particularly for lentiviral screens), as well as by the high cell number inputs, necessitating donor pooling and obscuring human variation. To address these concerns and enable genome-wide arrayed CRISPR screening in primary cells, we have developed a highly scalable, clog-free microfluidics platform that automates arrayed gene editing using electrostatic forces for liquid movement and the electric field for gentle payload delivery. This platform improves on existing electroporation technologies by leveraging unique cell droplet geometries that allow gentler gene delivery, improving primary cell function and viability. Here, show the results of an arrayed knock-out CRISPR screen in anti HER2 CAR-T cells, demonstrating a proof-of-concept system to optimize powerful next-generation adoptive cell therapies. We demonstrate the reduction of cell inputs from millions of cells per edit to >5,000 cells per edit, lower reagent costs by 10x and cellular inputs by 100-200x, while maintaining over 80% knock-out efficiency and no significant effect on viability. By reducing cell requirements, our system provides data at individual-donor resolution. The platform is easy to use, simple to scale to hundreds or thousands of genes, and is fully compatible with automation equipment performing downstream assays. Our platform will simplify and accelerate development of CAR-T therapies, making such optimization more accessible and affordable for experimental iteration. Refining CAR-T technology will equip cell therapies with improved cytotoxic specificity and tumor infiltration, as well as resilience against exhaustion, unleashing their full therapeutic potential. Rationally designed, highly targeted CAR-T may also achieve anti-tumor responses at lower doses, reducing toxicity in patients.

1458 Advancing NF1 Schwann Cell Targeted Therapy via Gene Regulatory Protein Development and Polymer Nanoparticle Discovery

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Introduction: Two key challenges remain for treatment of neurofibromatosis type 1 (NF1) patients: 1) development of a broadly applicable therapeutic approach for all NF1 patients, and 2) the ability to load and deliver large payloads (*i.e.*, CRISPR payloads >4.7 kb) to Schwann cells (SCs). To overcome these challenges, a multidisciplinary team from two different institutions is combining their payload and delivery vehicle expertise to develop a NF1 therapeutic formulation consisting of polymer nanoparticle (PNP) delivery vehicles and a DNA payload that will correct the underlying cause of NF1. This work is focused on creating targeted epigenetic regulatory proteins (ERPs) to be transient therapeutic payloads that cause persistent down-regulation of mitogen-activated protein kinases 1 and 2 (MEK1/2) and delivering plasmid DNA (pDNA) encoding these ERPs to SCs *in vitro* and *in vivo*. Methods: *Payload* - Bioinformatics tools, such as ChopChop, were used to design spCas9 guide RNAs (gRNAs) targeting the Map2k1 and Map2k2 genes. All gRNAs were chosen with the appropriate 3' NGG PAM sequence specific for *Sp*Cas9 and with varying distances in the promoter region upstream of each gene's transcriptional start site. To screen these 20 gRNAs for the strongest repressors of each gene, vectors expressing a single guide were co-transfected using Lipofectamine 3000 into Neuro2A cells. RNA expressed by pDNA was harvested, and qPCR was used to measure the repression capabilities of the different gRNA candidates. *Delivery vehicles* - PNPs were synthesized via reversible addition-fragmentation chain transfer polymerization, purified, and characterized via a proprietary design-build-test-learn PNP screening platform. PNPs were characterized for size and polydispersity using dynamic light scattering, loading efficiency via ribogreen fluorescence spectroscopy, and cytotoxicity and transfection efficiency using flow cytometry. Results: The "payload" team from the first institution designed, developed, and screened 20 gRNAs (*i.e.*, 10 Map2k1 Cas9 Guides and 10 Map2k2 Cas9 Guides). Two gRNAs were identified as promising lead candidates. For Map2k1, guide #7 of 10 showed the most promise, repressing Map2k1 expression by ~70%. For Map2k2, guide #1 of 10 showed the most promise, reducing Map2k2 expression by ~95%. In parallel, the "delivery" team from the second institution synthesized >500 PNPs with diameters of ~10 - 2,000 nm and polydispersity indices of ~0.1 - 0.57. Most PNPs showed loading efficiencies >90%,

and multiple PNPs yielded transfection efficiencies between 4-8% when tested on murine SCs *in vitro*. An indirect relationship was observed between the transfection efficiencies and cell viability values. Conclusions & Next Steps: Results suggest gRNAs for Map2k1 and Map2k2 can repress gene expression in neural cells, and future studies will seek to demonstrate these findings in SCs *in vitro*. Gene expression and MEK protein levels will also be measured over time. Initial PNP screening efforts successfully obtained size, loading efficiency, transfection efficiency, and cytotoxicity data that will be used by machine learning to enable improved PNP design and performance in future screens. PNPs will also undergo *in vivo* biodistribution screening using a proprietary barcoding platform technology to further inform future delivery vehicle designs.

1459 Hematopoietic Stem Cell Gene Therapy as a Novel Therapeutic Approach for Severe NOD2-Deficient Crohn's Disease

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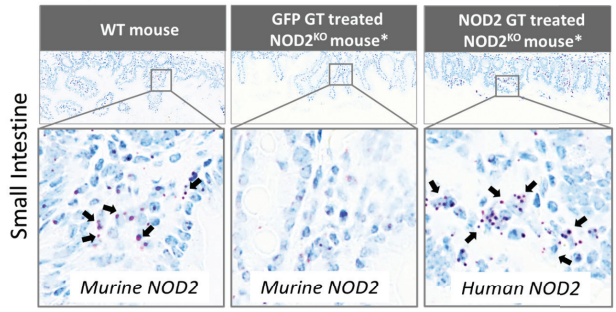
Deficiency of NOD2 (nucleotide-binding oligomerization domain containing protein 2) demonstrates the strongest genetic association to Crohn's inflammatory bowel disease (CD). Mounting evidence links NOD2 variants with poor clinical outcome, particularly in pediatric and early onset CD. CD patients with loss-of-function NOD2 polymorphisms, particularly carriers of more than one risk allele, frequently present an aggressive, fistulizing and fibrostenotic disease, requiring multiple surgical resections, and are more refractory to some available therapies. NOD2 signaling regulates gut innate immunity by sensing bacterial muramyl dipeptide (MDP) and fine-tuning Toll-like and Nod-like receptor (TLR/NLR) responses. NOD2-defective myeloid cells are implicated in CD pathogenesis, where ineffective clearance of bacterial infections drives a dysregulated, granulomatous inflammatory response. Here we present preclinical data on OTL-104, an autologous haematopoietic stem cell gene therapy (HSC-GT) which aims to stably restore NOD2 expression in gut resident macrophages, to correct the dysfunctional immune surveillance linked to NOD2-deficient CD pathogenesis. We used *in vitro* and *in vivo* models of NOD2 deficiency to evaluate the safety and efficacy of OTL-104 to restore NOD2 expression and function. NOD2^{KO} human myeloid cells differentiated *in vitro* from CRISPR-generated NOD2^{KO} CD34^{+} HSCs are unable to mount a proinflammatory cytokine response to MDP stimulation. Similarly, myeloid cells differentiated from CD34^{+} cells obtained from peripheral blood of genetically characterized NOD2-deficient CD patients, are also refractory to MDP stimulation and unable to generate a normal cytokine response profile (IL-8, TNFα, IL-6, CXCL1/2 and IL-10). Transduction with a lentiviral vector (LV) expressing NOD2 under the macrophage-restricted chimeric CathepsinG/cFES promoter fully restores NOD2-mediated IL-8 and TNFα responses in NOD2^{KO} CD34^{+} derived monocytes. In NOD2-deficient patient cells, LV transduction also restores MDP-induced cytokine responses to levels comparable to those observed in monocytes derived from CD34^{+} cells from healthy donors. Transplantation of lineage negative (*Lin*$^{-}$) hematopoietic stem/progenitor cells (HSPCs) transduced with the OTL-104 vector in NOD2^{KO} mice was used as an *in vivo* model of gene therapy for CD. Compared to wild-type mice, NOD2^{KO} mice fail to release systemic inflammatory mediators and recruit myeloid cells in response to MDP administration. Transplantation of transduced *Lin*$^{-}$ HSPCs restores MDP-induced systemic release of IL-6 and CXCL1 as well as innate mobilization of monocyte/macrophage cells. Transplanted NOD2^{KO} mice display normal hematopoiesis and stable vector copy numbers in hematopoietic cells. Key to the success of our therapeutic approach, histopathological analysis of intestinal *lamina propria* from transplanted mice shows a normal biodistribution and physiological NOD2 gene expression in tissue resident cells (Figure 1). These results confirm the negative impact of NOD2 deficiency in primary immune activation and support the therapeutic potential of OTL-104 HSC-GT for long-term correction of NOD2-deficient CD.

Figure 1. HSC gene therapy restores a normal intestinal biodistribution of NOD2 gene expression. Detection of NOD2 mRNA (*in situ* RNAScope) in intestinal lamina propria of wild-type (WT) and Gene Therapy (GT) treated NOD2^{KO} mice, confirms NOD2 gene expression is restored within hematopoietic derived tissue resident cells.



*Transplantation of gene modified NOD2^{KO} *lineage*$^{-}$ cells into NOD2^{KO} recipients following transduction with LV vectors to express GFP or human NOD2 (OTL-104 vector)

1460 Process and Platform Development for Production and Purification of CNS-Tropic Engineered AAV Capsids

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Recombinant adeno-associated virus (AAV)-based vectors for therapeutic gene delivery have shown great promise due to their favorable safety profile, broad tissue tropism, and ability to mediate stable transgene expression. Despite these advantages, conventional AAV serotypes exhibit limited delivery to the central nervous system (CNS) when employing less invasive routes of administration. An additional bottleneck to clinical translation is the design and optimization of manufacturing processes that enable large scale production and purification of AAV vectors. Modification of the AAV capsid can improve delivery efficiency and tissue specificity, however, these changes may also necessitate optimization of manufacturing process and purification conditions. In this study, we report the development of a scalable process for production and purification of engineered AAV capsids that show significantly enhanced CNS delivery relative to AAV9, the most commonly used vector for CNS gene therapy[1, 2].

Engineered AAV capsid variants were produced in suspension HEK293 cells using triple transient transfection where various production parameters including transfection reagent to DNA ratio, cell density, total DNA amount and transfection incubation time, as well as the production bioreactor systems were tested to determine the optimal AAV production process. Following AAV production, purification protocols for the different AAV capsid variants were developed using scalable affinity capture and ion-exchange column chromatography. Purification parameters such as pH and salt concentration were optimized for each engineered AAV capsid variant and for separating empty and full capsids. The optimized process demonstrates the manufacturing feasibility as well as product quality attributes that are critical to advancing novel genomic medicines to treat CNS disorders.
References: [1] Au H.K.E., Isalan M. and Mielcarek M. Gene Therapy Advances: A Meta-Analysis of AAV Usage in Clinical Settings. *Front. Med.* (2022) 8:809118. https://doi.org/10.3389/fmed.2021.809118 [2] Lykken, E.A., Shyng, C., Edwards, R.J. *et al.* Recent progress and considerations for AAV gene therapies targeting the central nervous system. *J Neurodevelop Disord* 10, 16 (2018). https://doi.org/10.1186/s11689-018-9234-0

1461 CAR T Cells Enable Superior Control of HIV Replication through Recognition of Abundant Antigens and Rapid Killing of Infected Cells

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Antigen-specific adoptive T-cell therapy has shown significant progress in treating different types of blood cancers, making it a promising therapy to limit the spread of chronically infected viruses such as HIV to reach a functional cure. Chimeric Antigen Receptor (CAR) and TCR αβ chains are the most common antigen recognition molecules engineered into T cells for redirection in adoptive T cell therapy. Although TCRs are shown to be >100 fold more sensitive than CARs, previous data from our lab has shown superior HIV suppression *in vitro* by CD4 extracellular-domain CAR-T cells compared to HIV-specific TCR-T cells, and the mechanism of CAR's superiority remains to be elucidated. Here, by demonstrating the timely induction of active-Caspase-3 within HIV infected cells by TCR and CAR-T cells, we show that CAR-T cells' superior control over HIV is a result of fast elimination of HIV infected cells. The reduced ability of HIV_{Pol} TCR to control HIV spread is due to low presence of pMHC antigen target, preventing elimination of infected cells before new virions are made and spread. Our research provides a mechanistic explanation for the superiority of CD4 CAR-T cells in vitro, highlights the difference between cancer and virus immunotherapy, and will inform novel TCR and CAR adoptive T cell therapies against HIV.

1462 Scalable Recombinant Adeno-Associated Virus (rAAV) Production in HEK293 Cells with the Use of a Histidine-Rich Peptide as Transfection Reagent

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Production of viral vectors in mammalian cells requires the delivery of foreign nucleic acids coding the gene of interest and necessary proteins involved in viral vector production. Most commonly, a transfection reagent (TR) is used to shuttle the nucleic acids into the cells. The choice of the transfection reagent for large scale manufacturing of viral vectors is governed by its efficiency, consistency, scalability, and cost. A suitable transfection reagent should meet several criteria to be a successful candidate for large scale viral vector production in mammalian cells. Stable DNA:TR complexes and low complexation volume are of special interest when scalability is considered. Currently, most of the commercially available transfection reagents lack one or more of the criteria mentioned above. Histidine rich peptides (HRPs) are known for their ability to deliver nucleic acids and proteins to target cells. However, to our best knowledge, their application to large scale viral vector production has not been reported. Here, we highlight the key benefits of the use of histidine rich peptide as transfection reagent in rAAV production process in HEK293 cells. These benefits include high transfection efficacy, industry leading rAAV yield with >1e12 Vg/mL, and scalability of transfection process facilitated by the stability of HRPs:DNA complexes and low HRPs:DNA complexation volume.

1463 A Cell-Based Drug Factory with Bioelectric Based Time and Dosage Control for Regulation of the Circadian System

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Background: Circadian rhythms are vital regulators of cyclical biological functions including metabolism and endocrine signaling. Dysregulation of the circadian system is a common problem faced by shift workers and travelers among others. Beyond the immediate detrimental impact to cognitive and physical function, disruption of the circadian rhythms are associated with negative health outcomes including increased risk of metabolic diseases, mental health disorders and some cancers. Light is a powerful regulator of the central circadian clock, however, alternative cues including metabolic and endocrine signals also play a role in regulating the circadian clocks and can be modulated therapeutically. An important challenge for regulating the circadian system is the need for a precisely timed therapy to influence the dynamic biologic process. Interfacing cell-based therapeutics with bioelectronic devices can enable timing and dosage control in addition

to cell health maintenance and monitoring. We utilized cells' innate ability to produce bioactive peptide therapeutics in combination with advances in synthetic biology to create a cell-based drug factory capable of producing metabolic and circadian rhythm modulating therapies on demand. **Methods:** We engineered a clinically relevant epithelial cell line to express endocrine signals demonstrated to regulate the circadian rhythm (leptin, and adrenocorticotropic hormone (ACTH)) under the control of a light responsive optogenetic system. We then validated these cells *in vitro* and characterized the productivity of the engineered cells in response to varying light intensity and time. Engineered cells were then encapsulated in silicone and hydrogel-based devices to isolate the therapy from the immune system and implanted in mice to evaluate *in vivo* therapeutic production. **Results:** Our results demonstrate that a clinically relevant cell line can be engineered to secrete therapeutically relevant peptides on demand. Engineered cells increased production of ACTH 34-fold and leptin 324-fold in response to light exposure. Further testing demonstrated this induction level is tunable based on light intensity and duration allowing for titratable therapeutic dosing. We determined that engineered cells can be turned on repeatedly over the course of days without a decrease in ACTH or leptin production, which allows for repeated, long term therapeutic dosing from a single engineered cell dose. When implanted in a mouse model, engineered cells remained viable and productive and were able to deliver the peptide therapeutic *in vivo*. **Conclusions:** In summary we demonstrated that clinically relevant cells can be used as cell-based drug factories to produce peptide based circadian regulators on demand. Therapeutic production can be timed and tuned based on user defined light input enabling precise dosing control. These cells have the potential to advance therapeutic circadian rhythm modulation. Additionally, due to its potential to produce any peptide based therapeutic this technology can function as a platform for the treatment of a wide array of indications.

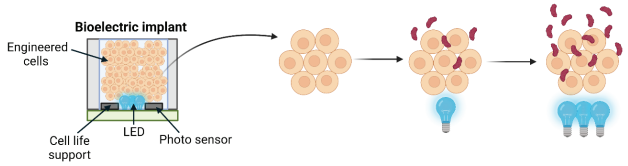

Figure 1: Overview of bioelectric implant and therapeutic dosing control

1464 A Comprehensive AAV Tropism Profile in Mice, Crab-Eating Macaques, and Marmosets

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Background: Recombinant adeno-associated viruses (rAAV) have become popular as gene delivery vehicles and development of gene therapy products. It is well-accepted that different AAV serotypes have different affinities for particular cell types. To make AAV-based gene delivery more accessible, several newly engineered AAV capsids were developed to target central nervous systems and muscles. However, comprehensive AAV tropism of wild-type AAV and new AAV serotypes among organs of different rodent and non-human primate (NHP) species remains unknown. **Methods**: 21 AAV serotypes, including AAV1-9, rh10, rh74, DJ, IE, LK03, B1, HSC15, PHP.B, PHP.eB, PHP.S, CAPB10, and Myo2A, containing CAG-H2B-tdTomato-barcode-WPRE constructs were mixed as an AAV pool and were intravenously injected to C57BL/6J mice, BALB/cJ mice, crab-eating macaques (Fig. 1A,B), and marmosets. Three weeks post injection, 20 tissues of mice and 29 tissues of NHPs were collected and examined for tandem-dimer tomato (tdTomato) expression and viral transduction efficiency. **Results**: The AAV transduction efficiency was found quite different across species. Although AAV-pool transduction was enriched in the glomerulus of the kidney and islet of the pancreas in mice, this sub-organ preference was not observed in NHPs. Besides, particular AAV serotypes showed different tropisms among species. For instance, AAV-PHP.b, PHP.eB and CAPB10 showed a high DNA transfection and RNA transduction in C57BL/6J mice, but low in BALB/cJ mice, and NHPs (Fig. 1C-E). In addition, even though PHP.S performed average in mice, the transduction efficiency of AAV-PHP.S showed high in most organs in NHPs (Fig. 1E). Since several AAV serotypes showed good transduction efficiency in some specific organs, a separate AAV serotype injection to mice or a smaller pool of AAV combination to NHPs was carried out as a confirmation. AAV4 exhibited significantly high DNA and RNA transduction among organs such as lungs, kidneys, and pancreas in mice and NHPs. Furthermore, AAV-PHP.S was broadly transduced among organs in NHPs when AAV4-tdTomato, AAV9-EGFP, and AAV-PHP.S-mBFP-HA were co-injected into NHPs. **Conclusions**: Our finding provides a comprehensive AAV tropism analysis based on barcode DNA and cDNA sequencing in C57BL/6J, BALB/cJ, crab-eating macaques, and marmosets. Additionally, our data characterizing AAV tropism in different species provides further guidance in engineering and use of gene delivery vehicles, which is applicable to AAV serotype selection for gene therapy to treat different therapeutic areas.

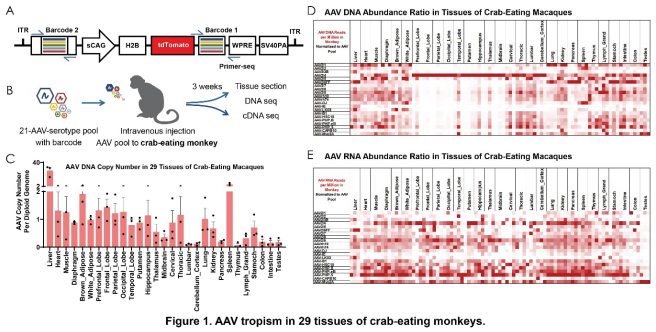

Figure 1. AAV tropism in 29 tissues of crab-eating monkeys.

(A) Constructs of AAV library containing two sets of 7nt-barcodes. **(B)** Schematic of intravenous injection of AAV pool to crab-eating monkey and AAV tropism analysis procedure. **(C)** AAV DNA copy number per diploid genome in 29 tissues of crab-eating monkeys (n=3). **(D)** Heatmap of AAV vector DNA ratio in 29 tissues of crab-eating monkeys. **(E)** Heatmap of AAV vector RNA ratio in 29 tissues of crab-eating monkeys three weeks post injection.

1465 Optimization of Packaging Efficiency for Adeno-Associated Virus by Evaluating Production Process Parameters Using Ambr 250® High-Throughput Platform

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The packaging efficiency (full to total capsid ratio, F/T ratio) for AAV is one of the critical quality attributes for the manufacturing and commercialization of AAV-based gene therapy. According to available literature and our data, production of AAV using the triple transfection method resulted in approximately 5 to 50 % of full AAV capsid in upstream crude harvest depending on serotype or gene of interest (GOI). The low quality of upstream crude harvest poses challenges for downstream purification. Previously, the lack of a reliable and cost-effective analytical tool to measure the F/T ratio on upstream crude harvest provided a major gap for upstream process development. ELISA-based approach coupled with genomic titer analysis is usually the method to estimate the F/T ratio for upstream crude harvest or other in-process samples. However, the data obtained by this approach are often unreliable and ambiguous due to low accuracy and high assay variations. Other more reliable analytical tools for F/T analysis, such as AUC (Analytical Ultracentrifugation) or negative staining TEM (Transmission Electron Microscopy), and cryo-EM (Cryogenic electron microscopy) are difficult to apply to upstream crude harvest due to low titer and high impurities in these samples while the cost for these assays is very high. Recently, several new analytical methods have been developed that can provide accurate and cost-efficient ways to analyze the F/T ratio for upstream crude samples. In this study, by using a Design of Experiment (DOE) approach, we sought to evaluate the impact of several production process parameters on AAV titer and packaging efficiency using the Ambr 250® High-Throughput Platform. We utilized our proprietary AAV packaging plasmids and AAV8-GFP as the study model. Titer and the F/T ratio on upstream crude samples were analyzed using the ddPCR titer assay and SEC-UV-MALS (Size-Exclusion Chromatography coupled with Ultraviolet and Multi-Angle Light Scattering detectors) analysis, respectively. After conducting two rounds of DOEs, we were able to significantly increase the full percentage of AAV-GFP in crude harvest by approximately 3-fold from the baseline. Additionally, we identified several production process parameters that played important roles in packaging efficiency for AAV8-GFP and built a strong model based on the DOE to predict an optimal condition for maximizing packaging efficiency for AAV8-GFP. In summary, this study demonstrates that we can significantly increase the AAV packaging efficiency at the upstream production stage, and a similar approach is ready to be implemented into upstream process development for different AAV serotypes and client-specific GOIs.

1466 Identification of Novel Inflammation-Inducible Promoters Using a Hybrid-Barcoded SuRE™ Library

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AAV-based gene therapy vectors are promising candidates for the treatment of inflammatory disease, resulting from a biological response of the immune system triggered by a variety of different factors. Regulatory elements, including promoters and enhancers, are engineered for use in AAV vectors to optimize the strength, kinetics, and specificity of transgene expression. The incorporation of promoters inducible by inflammation will help to reduce the risk for side effects due to overexpression of and/or continuous exposure to the anti-inflammatory therapeutic protein by such AAV vectors. The aim of this study was to identify inflammation-inducible hybrid-promoters and/or *cis*-regulatory elements that show improved inducibility and/or higher expression compared to the reference inflammation-inducible promoter NFkB-responsive CMV (NFkB-CMV promoter). To this end, the Survey of Regulatory Elements (SuRE) methodology was applied to identify new *cis*-regulatory elements in the human genome. A barcoded library containing around 300 million human DNA fragments/elements, with an average insert size of about 300 base pairs (bp), was generated and used to transfect HT1080 cells. Transfected HT1080 cells with and without stimulation with TNF alpha (TNFα) and IL-1 beta (IL-1β) human recombinant cytokines were analyzed for expression levels of the ~300 million elements as compared to their frequency in the input library to generate a genome-wide profile. Elements displaying high expression in stimulated conditions compared to unstimulated conditions were selected from the SuRE screening and combined with the reference NFkB-CMV promoter to generate a new barcoded library, consisting of ~40,000 new hybrid combinations, each of around 600 bp in size. The new barcoded hybrid-library was subsequently used in a second round of screening in HT1080 cells. The best performing hybrid-elements were selected for further analysis in the context of the AAV2 genome upon plasmid transfection and AAV transduction, with luciferase as reporter protein. Primary cells and different cell lines were used to determine the strength and inducible character of the new hybrid promoters. The expression profiles from plasmids and AAV viruses revealed a number of new hybrid promoter elements that displayed improved inducibility and/or higher expression under inflammatory conditions compared to the reference NFkB-CMV promoter.

1467 Engineered Virus-Like Particle to Deliver CRISPR-Cas9 Ribonucleoprotein with Minimal Off-Target Effects

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Gene editing using the CRIPSR-Cas9 system is promising to treat many genetic diseases with unmet needs. Transient delivery and prompt editing of the CRISPR-Cas9 system is highly desirable with the goal to minimize its genome off-target effects, especially when delivered in vivo systematically. The development of engineered virus-like particle (eVLP) systems to deliver Cas9 ribonucleoprotein (RNP) that allows for rapid genome targeting as well as degradation of the Cas9 protein is therefore promising to control the off-target effect of CRISPR-Cas editing systems. Here we engineered a VLP containing the wildtype SpCas9 and a *TTR* gene targeting sgRNA. We evaluated the off-target effect of the SpCas9-*TTR* eVLP and compared it with the liposome-based CRISPR RNA. Two methods were used for genome-wide off-target evaluation, namely the GUIDE-Seq and a method for editing event detection (EDITED-Seq). Briefly, we recently developed the EDITED-Seq method for simultaneous identification and verification of new, known, and predicted potential off-target sites, and is compatible with regular gene editing protocols without extra cell manipulations. To produce the SpCas9-*TTR* eVLP, four plasmids (VSV-G, MMLV gag-pol, MMLV gag-SpCas9 and *TTR*-targeting sgRNA plasmids) were co-transfected into LentiX-293T cells. At 72h post-transfection, the supernatant was harvested, purified, and concentrated 100-fold. The eVLPs produced (50 uL) were then used to transduce HEK293T and HepG2 cells in 24-well plates (pre-treated with dsODN for GUIDE-Seq analysis). Similarly, liposome-based RNA (at 15 nM sgRNA concentration and a weight ratio of sgRNA:mRNA being 1:1) transfections targeting the same site were conducted on both cell lines. At 48h after transduction or transfection, cell genomic DNA was harvested, and 400 ng DNA was used for next-generation sequencing for GUIDE-Seq and EDITED-Seq analyses. The GUIDE-Seq results showed that the eVLP method had no detectable off-targets in both cell lines (with the on-target site showing thousands of unique GUIDE-Seq reads), whereas the liposome-based RNA method showed several off-target sites (5 to 7 among triplicates). The EDITED-Seq showed consistent results with GUIDE-Seq, except that one off-target site was detected from the eVLP method samples. This off-target site was the top off-target site (with the most abundant GUIDE-Seq reads) detected in the liposome-based RNA method. The data provides genome-wide unbiased evidence that using eVLP to deliver CRISPR-Cas9 ribonucleoprotein may substantially reduce off-target effects than the liposome-based RNA delivery, likely due to faster degradation of the RNP complex. Further studies on more targets, more cell lines and in vivo settings are needed to fully characterize the potential minimal off-target effects associated with eVLP delivery.

1468 SLS-009 Clears Only Mutant Huntingtin Aggregates in a Severe Huntington's Disease Mice Model

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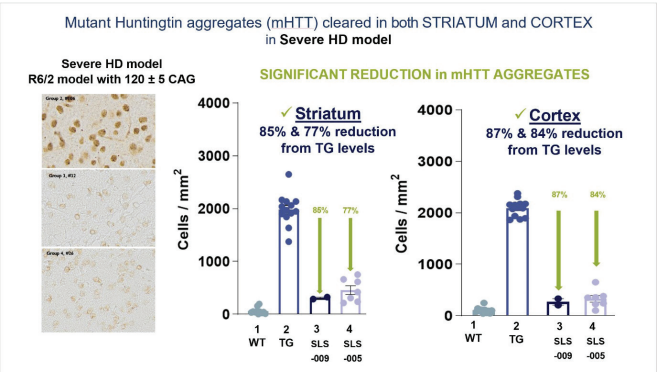
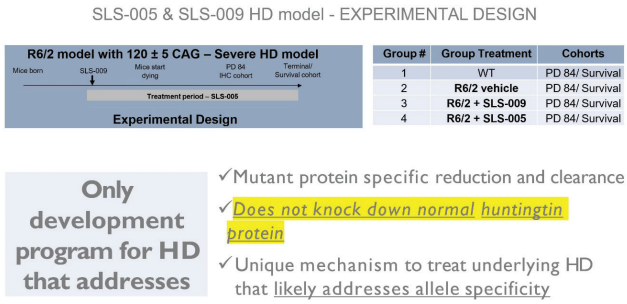
Huntington's disease (HD) is a fatal dominantly inherited neurodegenerative disorder caused by CAG repeat expansion (> 36 repeats) within the first exon of the huntingtin gene. Although the huntingtin protein is ubiquitously expressed, the neuropathology of HD is characterized by progressive decline in motor, cognitive and various psychiatric functions involving striatum and cortex regions of the brain. There is currently no disease-modifying treatment. The severe juvenile form of the disease with > 60 CAG repeats has the closest resemblance to the R6/2 HD rodent model utilized here with ~ 120 CAG repeats. This is a severe rodent phenotype that rapidly progresses and has the distinct mutant Huntingtin (mHTT) aggregates present inside cells in the striatum and cortex regions. Upon delivery of two therapeutic agents, SLS-005 (administered starting on Day 31) and SLS-009 (also administered on day 31) to two separate groups of R6/2 rodents, along with two additional groups of wildtype and transgenic controls (positive and negative controls) resulted in significant reduction of the mHTT aggregates in striatum and cortex regions by Day 84. We quantified an 85% and 77% reduction with SLS-009 and SLS-005, respectively, in mutant aggregate counts in the striatum and an 87% and 84% reduction respectively in the cortex, compared to the diseased animals. Corresponding biomarker, DARPP-32, supported the above findings with reductions of similar magnitude. Trehalose has been described in the past in several in-vitro and in-vivo experiments to have benefits in HD and other neurological disorders. However, the past doses and administration paradigms were not uniform and led to mixed observations in the literature. Here, we demonstrated overcoming the previous limitations with new formulations (SLS-005 and SLS-009) and appropriate therapeutic dosing to elicit disease-modifying benefit in two critical regions of the brain in this severe R6/2 HD model. The therapy is currently believed to be the only one in development for HD treatment that addresses the clearance of mutant huntingtin protein alone and is potentially allele-specific, as the wild-type huntingtin protein is unaffected through the unique mechanism of action of SLS-009 and SLS-005.





1469 A Novel, Chemically-Defined, Serum-Free Cell Culture Medium for Expansion of Transfected T Cells

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T cell therapies require ex vivo culture and expansion of cells at scales relevant for therapeutic applications. Human AB serum (HAB) or fetal bovine serum (FBS) have been key components of the cell culture media used in T cell therapy manufacturing processes. Although these components assist in optimal growth and improved gene transfer efficiencies, they have inherent variability and supply challenges. In addition, due to animal origin and traceability issues, they have a potential of introducing pathogens to the process. Without the addition of HAB or FBS, T cells, especially when patient derived, fail to grow adequately and exhibit reduced efficiencies of gene transfer, irrespective of the transfection method applied. In the transfection arena, electroporation offers a powerful non-viral delivery method for T cell engineering. In this study, we present data generated by utilizing a novel, chemically-defined, animal component free cell culture medium that robustly expanded human T cells in the absence of serum and worked well in combination with electroporation as demonstrated by high transfection efficiency and cell proliferation. Data were generated using activated T cells obtained by stimulating CD3 cells or PBMC. The recovery, proliferation and expansion profiles of the cells post transfection demonstrate efficiency and consistency of the electroporation technology utilized. These results suggest that the new chemically-defined, serum-free medium along with the electroporation technique employed can support scalable GMP manufacturing of clinical-grade T cell therapies.

1470 Primary CD4 T-cell Biofactory for Antigen-Inducible *In Situ* Synthesis of Engineered Proteins

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Toxicity associated with systemic delivery of drugs limits its application for diseases that evolve *in vivo* (e.g. cancer, autoimmune diseases and viral infections). T-cell Biofactory is an engineered cell-based platform that capitalizes on T-cell's innate extravasation capability and transforms cells into a living vector that assesses the disease burden and synthesizes calibrated amounts of complex protein-based biologics with desired properties at the disease site. This offers the potential to exert a broad range of functions without any dose misalignment, which otherwise causes side effects. Clinical translation of this technology has however been impeded by insufficient expression of the biologics from the T cell and manufacturing bottlenecks specific to the T-cell products. Compared to the pan T cells, the CD4 T cells offer to overcome these challenges due to their propensity for higher transduction, faster expansion, and a more productive transcription machinery. The results presented herein show that capitalizing on the CD4 T-cell biology, the CD4 T-cell Biofactory, compared to its CD8 counterpart, when configured with chimeric antigen receptor (CAR), transduces ~3-fold better, expands ~2-fold more, induces biologics ~5-fold more, and exhibits minimum cytolytic activity. Additionally, we also report on a scalable, simple yet efficient method for manufacturing primary human T-cell Biofactories encoded with large genetic payloads. Altogether, the correctly configured CD4 T-cell Biofactory is a cell-based system that may synergize with a reduced dose of other directly acting therapies to exert desired efficacy while mitigating any morbidity associated with its overdosing.

1471 A Mutation Agnostic Hematopoietic Stem Cell GeneTherapy for Metachromatic Leukodystrophy

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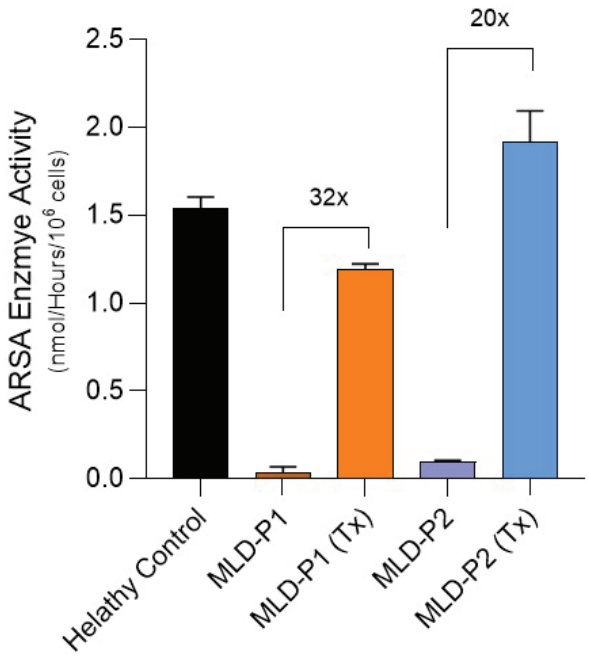
Metachromatic leukodystrophy (MLD) is a rare genetic disorder caused by mutations in the Arylsulfatase-A (ARSA)gene. The enzyme plays a key role in sulfatide metabolism in brain cells, and its deficiency leads to neurodegeneration.The clinical manifestations of MLD include stagnation and decline of motor and cognitive function, leadingto premature death with limited standard treatment options. Here, we describe a mutation-agnostichematopoietic stem and progenitor cell (HSPC) gene therapy using CRISPR-Cas9 and AAV6 repair template asa prospective treatment option for MLD. Our strategy achieved efficient insertions and deletions (>87%) anda high level of gene integration (>47%) at the ARSA locus in human bone marrow-derived HSPCs, with no detectableoff-target editing. As a proof of concept, we tested our mutation-agnostic therapy in HSPCs derivedfrom two MLD patients with distinct mutations and demonstrated restoration of ARSA enzyme activity (>30-fold improvement) equivalent to healthy adults. In summary, our investigation enabled a mutation-agnostic therapyfor MLD patients with proven efficacy and strong potential for clinical translation.



1472 Evaluating the Permissibility of Human iPSC-Derived Cell Types to AAV Transduction as an *In Vitro* Model for Gene Therapy

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INTRODUCTION: The differentiation of induced pluripotent stem cells (iPSC) into specialized cell types of the human body represents a major advancement for the development of biologically relevant disease models. Generation of patient-specific cell models for in vitro testing has cultivated the concept of "disease-in-a-dish" for iPSC-based disease modeling. These model systems are increasingly being used to evaluate various gene targeting approaches, including adeno-associated viral vectors (AAV), to directly correct the genetic defect or mutation. The use of AAV for gene therapy has recently become a clinical reality for the treatment of spinal muscular atrophy (SMA) and a rare form of blindness. To identify relevant iPSC-based in vitro models to support the analytical characterization of AAV gene therapies, the permissibility of different iPSC-derived cell lines to specific AAV serotypes needs to be defined. This requires the establishment of an efficient protocol for the AAV transduction of a particular iPSC-derived cell type, for which several aspects need to be optimized, including viral vector serotype, multiplicity of infection (MOI), choice of promoter, transduction media, and timing of transduction. **METHODS:** Human iPSC-derived cell types included commercially available iCell® Cardiomyocytes (iCell CM) and iCell Retinal Pigment Epithelial Cells (iCell RPE) from FUJIFILM Cellular Dynamics, Inc. (FCDI). Cryopreserved cells were thawed, plated, and maintained in culture according to the manufacturer's recommendations until the time of transduction. AAV viruses engineered to express green fluorescent protein (GFP) were prepared internally or obtained commercially. Different methods to evaluate transduction efficiency included standard fluorescence microscopy to visualize GFP-positive cells, flow cytometry (CytoFLEX, Beckman Coulter) to quantify green cells, and Celigo image cytometer (Nexcelom) to both obtain images and quantify GFP in situ. **RESULTS:** iCell CM and iCell RPE were transduced with AAV1, -2, -4, -5, -6, -8, and -9 in standard maintenance medium and serum-free medium on days 7 and 10 post-thaw for iCell CM and days 14 and 21 post-thaw for iCell RPE. AAV6 showed the highest transduction efficiency in iCell CM, with AAV2 also working well. AAV2 show high transduction efficiency in iCell RPE. However, the capability of other AAV serotypes to transduce iCell RPE remains to be tested. In general, transduction on the earlier timepoint, day 7 for iCell CM and day 14 for iCell RPE, worked better than on the later timepoint, day 10 for iCell CM and day 21 for iCell RPE, with some exceptions. Moreover, the CAG promoter drove higher transgene expression than CMV. In addition to vector tropism, comparison of iPSC-CM and iPSC-RPE show that cell type significantly affects transgene expression. **CONCLUSION:** Human iPSC-derived cells can be efficiently transduced with AAV. The systematic exploration of the numerous variables that impact the efficiency of AAV transduction for any given cell type is required. Cryopreserved cells from commercial sources that are manufactured reproducibly in scale are an ideal reagent for use in these studies because of their "thaw-and-go" consistency. Proper handling and storage of AAV material is critically important as it can affect the transduction efficiency. Finally, the combination of iPSC derived cells and AAV technology to modulate in vitro cellular disease models for pre-clinical studies and potentially even potency release assays is a powerful system that is now available as part of a drug manufacturing process for material release.

1473 Novel Peptide Sequences to Fuse to Iduronate-2-Sulfatase for the Treatment of Mucopolysaccharidosis Type II

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Mucopolysaccharidosis Type II (MPSII) is a lysosomal storage disorder caused by a deficiency in the Iduronate-2-Sulfatase (IDS) enzyme due to a mutation in the *IDS* gene. The resulting accumulation of glycosaminoglycans (heparan sulfate (HS) and dermatan sulfate (DS)) leads to a multisystem disease with neurodegeneration in two thirds of patients. Enzyme replacement therapy is ineffective at treating neurological disease, as the blood-brain-barrier (BBB) prevents the entry of intravenous enzyme into the brain. Haematopoietic stem cell transplant is also unsuccessful, presumably due to insufficient enzyme production from transplanted cells engrafting in the brain. The fusion of peptides to lysosomal enzymes has the potential to increase the amount of enzyme reaching the brain from the periphery by enabling greater uptake of enzyme into the brain. We have previously demonstrated that a haematopoietic stem cell gene therapy (HSCGT) strategy, using a lentivirus containing IDS fused to ApoEII (LV.IDS.

ApoEII), can correct neuropathology in MPSII mouse models to a greater extent than IDS alone. Here we present data using two other peptides, RVG (rabies virus glycoprotein) and gh625 (Herpes virus glycoprotein derived peptide), fused to IDS and delivered via HSCGT in MPSII mice. Both peptides have been shown to increase delivery of drugs across the BBB into the brain. There were encouraging reductions in pathology with both IDS.RVG and IDS.gh625, however enzyme levels in the brain were lower than ApoEII (<20% of ApoEII for both). Also, both IDS.RVG and IDS.gh625 constructs failed to completely normalise inflammation and lysosomal swelling in the brain. We have therefore also screened several other peptide constructs (IDS.HS2 and IDS.HS5) for their ability to increase uptake into the brain using an *in vitro* BBB uptake assay. HS5 was able to improve BBB uptake over ApoEII (65% increase) and also improved uptake into multiple cell types including cardiomyocytes (37%), retinal pigment epithelium (105%) and osteoblasts (116%). HS5 also increased transcytosis across BBB cells over native IDS and to the same extent as ApoEII. We believe that HS5 may, therefore, may prove to be a viable candidate for stem cell gene therapy for Mucopolysaccharidosis Type II.

1474 CRISPR/dCas9 Based Epigenetic Manipulation of Cancer Cell Line HepG2 for Modulation of Epithelial to Mesenchymal Transition

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Epigenetic mechanisms are critical in communication between environment and genome, thus playing an important role in many diseases, including cancer. Molecular tools have been developed for targeted and specific manipulation of epigenetic modifications, including the CRISPR/dCas9 system, that enables studying interplay of different epigenetic marks and their effects on gene expression. Fusion of different epigenetic modifiers to the inactivated dCas9 allows for introduction of desired epigenetic modification in the genome region of interest. In this study, we investigated interactions between DNA methylation and histone modifications, and the effects of these epigenetic marks on the activity of two genes, *ZEB1* and *SNAI1,* coding for transcription factors involved in epithelial to mesenchymal transition (EMT). To achieve this, we used the DNMT3A-dSpCas9 fusion for targeted DNA methylation in combination with dCas9 fusions with different histone modifying effectors domains: G9a-SET for induction of H3K9 mono and di-methylation, G9a-Y1154F-SET for H3K9 trimethylation, LSD1-SET for H3K4me1/2 demethylation, and the catalytic domain of KDM5a for H3K4me3 reduction. Multiple gRNAs were used to target the entire CpG islands of *ZEB1* and *SNAI1* genes in the HepG2 cell line. A synergistic effect of the G9a-dSpCas9 and DNMT3A-dSpCas9 was observed on both *ZEB1* and *SNAI1* gene expression, while the synergy effect on DNA methylation was observed only in *SNAI1* gene promoter. Interestingly, none of the effector domains themselves were able to increase DNA methylation level. However, in combination with DNMT3A, domains G9a, G9a-Y1154F and LSD1 increased DNA methylation level on one or both targeted genes, in comparison to DNMT3A combined with the inactive version of the domains. Only KDM5a did not affect CpG methylation when combined with DNMT3A, but still showed synergistic effect on the expression level of both genes. When DNMT3A-dSpCas9 was combined with either G9a-Y1154F or LSD1, it too showed synergistic effect on transcriptional silencing of the targeted genes. These manipulations resulted in decrease of the protein levels of targeted genes, and changed expression of EMT markers, CDH1 and CRB3. The results obtained in this study suggest that different epigenetic modifications (cytosine methylation and histone marks) operate in synergistic manner, on both the expression of selected genes, and DNA methylation. Ongoing research will provide more mechanistic insights into understanding the relationship between DNA methylation and histone modifications.

1475 Process Improvements to Enhance AAV Recovery

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Sanofi, Waltham, MA

Improving purification recovery of AAV products is an industry-wide goal; however, this is particularly challenging for AAV serotypes that are prone to aggregation due to their physicochemical properties. The aggregation propensity impacts product stability at various process conditions and adversely impacts the recovery. This presentation will discuss the process development challenges associated with AAV aggregation and the opportunities identified to alleviate the impact on AAV recovery. In AAV purification processes, affinity chromatography with low pH elution is commonly used. However, low pH conditions with extended hold time were demonstrated to promote high levels of aggregation, which impacted subsequent unit operations. To mitigate such risks, an in-situ neutralization strategy was developed to control the aggregation levels. In addition, the anion exchange chromatography was optimized to remove aggregates, and the location of the virus filtration step was optimized to further improve process recovery.

1476 Base Editing of Hematopoietic Cells Restores Immune Function in a Mouse Model of Familial Hemophagocytic Lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis (FHL) is an immunohematologic disorder caused by hyperactivated T cells and macrophages that lead to severe inflammation and multiorgan damage. The uncontrolled immune activation is a result of impaired lysis of antigen-presenting cells due to defective cytolytic activity of T cells and NK cells. The disease-causing mutations in FHL type 3 (FHL3), which accounts for one third of FHL cases, are found in the *UNC13D* gene. Given that current treatment protocols, including allogeneic hematopoietic stem cell (HSC) transplantation, still show high mortality, novel treatment options are needed. As a proof of concept, we developed a base editing strategy to amend the disease-underlying mutation in *Jinx* mice - a preclinical model for FHL3. We generated several adenine (ABE) and cytosine base editors (CBE) to target the cryptic splice donor site in *Unc13d* intron 26. Screening in specifically generated reporter cells resulted in functional correction of 65-96% of cells. The best-performing base editors were applied to primary hematopoietic cells. To this end, ABE/CBE-encoding mRNA and corresponding guide RNAs were transferred to *Jinx* CD8+ T cells and HSCs by electroporation. While the ABE was not active in primary cells, 61-71% of *Unc13d* alleles were edited by CBE. Functional assays confirmed reestablished cytolytic activity of edited T cells. High-throughput sequencing-based CAST-Seq and rhAmp-Seq analysis to characterize CBE-associated off-target effects in *Jinx* mouse CD8+ T cells and HSCs revealed unexpected genotoxicity. We detected CBE-mediated chromosomal translocations as well as insertion/deletion mutations at both on- and off-target sites. The *Unc13d*-edited HSCs have been transplanted into conditioned *Jinx* mice to demonstrate functional restoration of lymphocyte cytotoxicity in the preclinical FHL3 model. The results of these *in vivo* experiments as well as the evaluation of off-target effects in *Jinx* mice transplanted with CBE-edited HSCs, will be presented.

1477 Non-Canonical NFKB Signaling Endows Suppressive Function through FOXP3-Dependent Regulatory T Cell Program

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Regulatory T cells (Tregs) is playing a central role in controlling adaptive immune response in human and mice. FOXP3 is a master transcription factor for Treg differentiation and suppressive function. Nuclear factor 'κ-light-chain-enhancer' of activated B cells (NFKB) signal is an essential signaling pathway related to the cell proliferation, differentiation and apoptosis among different lineages across the species. To date, functional roles of the non-canonical NFKB signaling in Tregs have been mainly studied in the transgenic mice, however the biological role of the non-canonical NFKB signaling in human Tregs is not yet fully discovered. To understand the biological role of non-canonical NFKB signaling in human Tregs, Treg-like cell line (MT-2 cells) and human primary Tregs are genetically engineered by CRISPR/Cas9. Interestingly, NFKB2 knockout MT-2 cells showed the downregulation of FOXP3, not by the NFKB1 knockout. In addition, the mRNA expression of FOXP3-dependent molecules including CTLA-4, GITR and IKZF-2 were significantly reduced by NFKB2 knockout MT-2 cells. Similar to the MT-2 cells, NFKB2 knockout human Tregs showed the significant reduction in the FOXP3 expression. Furthermore, NFKB2 knockout human Tregs showed downregulation of FOXP3-dependent molecules including CTLA-4, GITR and IKZF-2 similar to the Treg-like cell line. In addition to the phenotypic changes, NFKB2 knockout Tregs showed a reduced suppressive function compared to the wild-type and NFKB1 knockout Tregs. Here, we have shown that non-canonical NFKB signaling maintained Treg-like phonotype and suppressive function in human Tregs through the FOXP3-dependent regulatory T cell programme.

1478 AAV Gene Therapy for Autosomal Dominant Optic Atrophy Caused by Mutation in the Opa1 Gene

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Autosomal dominant optic atrophy (ADOA) is caused by mutations in the Opa1 gene. Opa1 is a dynamin-related GTPase that localizes to the mitochondrial membrane. Opa1 protein stabilizes the inner mitochondrial membrane and acts in mitochondrial fusion and inner membrane remodeling. Mutant phenotypes present with a progressive loss of retinal ganglion cells that results in optic nerve degeneration and legal blindness with a loss of visual acuity, optic disc pallor, and color vision deficits. Approximately 50% of pathogenic mutations result in a truncated protein due to mutations in the GTPase domain resulting in a dominant-negative effect. There are 8 different splice variants, with variant 1 (main variant) and 7 (E5B) being the most prevalent and consisting of the L-isoform and either the ΔS1 or both the ΔS1 and ΔS2 isoforms, respectively. Variant 5 lacks the L-isoform and only contains the ΔS1 isoform and is cleaved from the membrane and therefore soluble. Here we constructed an AAV-vectored gene

therapy for ADOA expressing normal human Opa1 and delivered with the AIM® library capsid AAV204. Expression of isoform variants 1, 5, and 7 were assessed in Opa1 knockout mouse embryonic fibroblasts (MEFs) with both un-tagged and Flag-tagged constructs. We further characterized Opa1 expression in both the wild-type (WT) and Opa1 heterozygous (HT) mouse retina 2 months post treatment with an intravitreal injection. Opa1 homozygous mice are embryonic lethal and therefore HT animals were used for Opa1 viral expression. Methods: AAV204 viral constructs were made for variants 1, 5 and 7 for both the un-tagged and Flag-tagged constructs. WT and Opa1 KO MEFs were transduced with all 6 constructs and analyzed 1-week post-transduction for RNA and protein expression. Next, 1 µL of 3.0×10^9 vg/µL AAV204-hOpa1 constructs were intravitreally injected into mouse retinas at 1 month of age and analyzed by RNA and protein expression at 2 months post-injection. Results: In cell culture, all 3 variants expressed both RNA and protein with each variant corresponding to the appropriate cleavage pattern on manual westerns for both the un-tagged and Flag-tagged constructs. Following transduction of the Flag-tagged construct in WT mice, variants 1 and 7 expressed protein, with variant 7 transcript only generating the L-isoform. However, there was no expression of variant 5 at either the RNA or protein levels. HT mice were thus transduced with only variants 1 and 7, with both expressing RNA and protein. Conclusions: These results suggest that AAV204-CBh-Opa1 expression is significant at both the RNA and protein levels both in vitro and in vivo. This indicates that intravitreal injection is an efficient route of administration for a gene therapy for Opa1 replacement in ADOA. There are ongoing studies to assess for long-term rescue of Opa1 in animals that were treated for 10 months with in vivo analysis for Optical Coherence Tomography, Electroretinogram, and Optokinetic Response in addition to RNA and protein expression analysis.

1479 Smartphone-Based Titration of Baculoviral and AAV Vectors

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Baculoviral expression systems are well-established in the manufacturing of diverse proteins, including viral vaccines and gene therapy vectors, such as AAV, in which yields can be as much as 10-fold higher than with traditional 293-based methods. The advantages of the baculoviral expression system include flexible product design, manufacturing speed and scalability, and inherent safety. Recombinant baculovirus is constructed by first cloning the gene of interest (GOI) into a transfer plasmid. Subsequently, this transfer plasmid is co-transfected with a linearized parental AcMNPV genome into insect cells where homologous recombination takes place to generate a recombinant baculovirus. Successful expression of a recombinant protein relies on knowing the infectious titer of the baculovirus preparation, as it permits calculation of the multiplicity of infection (MOI) which can influence the final expression level of the recombinant protein. Plaque assays, endpoint dilution assays, quantitative real-time polymerase chain reaction, and flow cytometry, have all been used to determine baculovirus titers. However, these methods are time-consuming and labor-intensive, with time to results ranging anywhere from two hours to four days. In this work, we present an iOS and Android-compatible smartphone application that analyzes a gp64-specific lateral flow assay and can deliver plaque-forming unit values (PFU/ml) in 10 minutes when a reference virus with a known infectious titer is used. The simplicity of the assay facilitates easy monitoring and optimization of baculoviral production processes to ensure consistency and confidence in downstream applications. The two-step assay consists of adding a small amount (20µl) of baculoviral supernatant to the lateral flow device followed by imaging and analysis of the results using a smartphone. Densitometric analysis of the observed bands is performed by the intuitive GoStix Plus software that compares the results to an automatically downloaded, lot-specific standard curve. The result is a GoStix Value (GV) that, like a plaque or qPCR assay, can be used to normalize virus stocks before being used for the expression of a recombinant protein. Beginning with the construction and transfection of a ZsGreen1 expressing baculoviral vector, we were able to detect baculovirus as early as 4 days post-transfection (P0). In addition, we were able to demonstrate that these tests can be used to screen viral clones (plaques) for titer after one round of amplification (P1). The tests demonstrated titer values within 3-fold of current titration methods with coefficients of variation of less than 15%. Furthermore, to add to the utility of these lateral flow-based tests, we were also able to demonstrate the ability to detect and quantify baculovirus-produced AAV particles using an AAV capsid-specific antibody. This method will be the focus of our work moving forward. In summary, this highly convenient, lateral-flow-based titration technology can quantify both baculoviral and AAV vector preparations in approximately 10 minutes, reduce expenses related to labor and materials, and accelerate baculoviral vector production and protein expression workflows.

1480 Multiple Cell Processing Applications Using the Counterflow Centrifugation Technology

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Counterflow centrifugation technology utilizes cell separation based on their size and density. Here, we demonstrate flexibility of the technology to incorporate different cell processing methods, providing an opportunity for users to choose a method based on their specific cell therapy workflows. Currently there are several manual methods to isolate PBMCs from leukaphresis products but most of them are highly user-dependent and laborious. Our data demonstrate that the automated counterflow centrifugation technology using the CTS Rotea System can perform PBMC isolation methods in a highly reproducible and effective manner by comparing the performances of the counterflow centrifugation protocols and a density gradient manual method. The automated counterflow centrifugation methods included Ficoll, ACK lysis buffer, and red blood cell elutriation. Regardless of the processing methods, high percentage (>98%) of the leukocyte population was isolated with minimal loss in recovery and viability (Figure 1). In addition, cellular compositions and post-processing effects of the isolated cells were characterized and compared. In all, the data show effective and consistent PBMC isolation across the four methods and flexibility of the automated counterflow centrifugation

system to successfully integrate existing methods. This work can serve as basis for expanding cell processing applications by leveraging the existing technology.

Cell types	Gating strategy	Frequency (%) of cells				
Pre-processing	Ficoll	Manual Ficoll	Lysis	RBC Elutriation		
Leukocytes	CD45+	79.7	99.6	98.5	99.7	99.8
T cells	CD45+, CD3+	28.5	31.4	25.9	27.1	33.5
B cells	CD45+, CD19+	7.63	9.08	8.69	7.13	8.28
Monocytes	CD45+, CD14+, CD16-	13.4	13.2	16.1	19.6	14.5
Neutrophils	CD45+, CD14-, CD16+	9.15	7.13	8.85	7.53	7.0
NK cells	CD45+, CD56+	9.77	6.67	8.35	8.76	8.54
Dendritic cells	CD45+, CD11c+	35.9	26.9	36.6	39.4	30.1
Platelets	CD41a+	37.4	21.7	37.4	29.8	26.9
Red blood cells	CD235a+	10.7	4.66	3.69	2.96	4.74

Figure 1. Cellular compositions of PBMC subpopulations defined by flow cytometry.

1481 High Throughput Discovery of Optimized Human Genomic Regulatory Elements That Selectively Decrease Off-Target Expression in Dorsal Root Ganglion and Liver in Mice and Non-Human Primates

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Adeno-associated virus (AAV)-mediated gene therapy (GT) has demonstrated transformative potential in treating genetic disorders, but off-target transgene expression remains an important safety issue. Encoded's next generation sequencing (NGS)-based functional screening and model-based sequence enrichment platform efficiently identifies regulatory elements (REs) from human genomic sequences to modulate transgene expression. Previously, we identified and validated 3'UTR elements that selectively reduced off-target liver expression to undetectable levels while maintaining central nervous system (CNS) expression in mice. We also applied our high-throughput screening method to achieve a specific transgene expression profile: selective de-targeting of dorsal root ganglion (DRG) expression, while maintaining CNS expression. We simultaneously tested >10,000 genomic REs and identified and validated top 3'UTR genomic sequences that achieved robust DRG de-targeting and maintained CNS expression in mice. Here, we used this large-scale functional data for predictive modeling to identify sequence features that contributed to DRG de-targeting and designed second-generation libraries (GEN2) to further improve performance. Using these GEN2 AAV libraries, we developed RE sequences that simultaneously de-target expression in DRG and liver, discovered novel candidate DRG de-targeting sequences, and achieved further improvement in de-targeting efficiency. Finally, multiplex GEN2 library screening in non-human primates (NHPs) showed conserved function across species for top de-targeting REs. In conclusion, we applied high-throughput functional and computational screening to efficiently discover, optimize, and validate multiple de-targeting REs to drive potency and specificity in cell types and organ systems, including DRG neurons and liver. These human genomic REs have the potential to improve gene therapy constructs and may enable optimized safety and efficacy profiles for patients through increased flexibility in GT modality, dose, and route of administration.

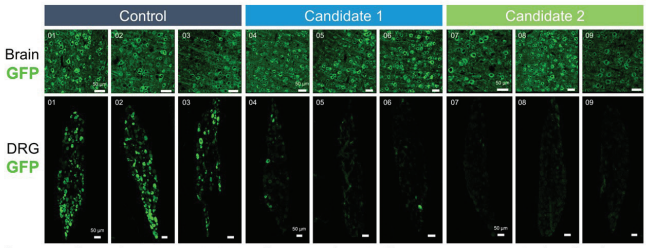

Figure 1. DRG de-targeting: Representative images of mouse samples stained for AAV transgene expression show that 3'UTR sequence elements substantially reduce off-target expression in the DRG, while maintaining CNS expression.

1482 Self-Complementary AAV Genome Orientation May Affect Overall Transgene Expression Efficiency

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Adeno-associated virus (AAV) gene-therapy has expanded prolifically in the past decade, with over 150 active clinical trials currently registered through the NIH database. While great progress has been made in novel capsid engineering, many of the same intrinsic limitations of AAV remain, particularly the limited transduction efficiency, immunological interference and a limited genomic capacity of ~4.7kbp. Previous research has demonstrated that transduction efficiency can be improved by employing modifications to the inverted-terminal-repeats (ITRs) of AAV, forcing it to package as a self-complementary form (scAAV) instead of its typical single-stranded form, thus bypassing second-strand synthesis during vector transduction. This is done by modifying the terminal-resolution-site (TRS) and the D-sequence on one ITR (ΔITR), but not the other, disallowing hairpin resolution but maintaining packaging viability. This intrinsically reduces the packaging capacity by half, but remains an option for many monogenic treatment strategies. Interestingly, there are no studies found by the author that demonstrate if there are any differences between placing the ΔITR is upstream of the transgene (ΔITR-up) or downstream of the transgene (ΔITR-down). In this experiment, an AAV2-ITR vector

plasmid with an enhanced green-fluorescence protein (EGFP) driven by a chicken-β-actin promoter (CBAp) was packaged ΔITR-up, and again with ΔITR-down (Figure 1) using similar packaging (HEK293, triple-plasmid transfection) and purification methods (Iodixanol & dialysis spin columns). The same process was setup for AAV3-ITR carrying the same genome payload. Huh7 cells were transfected with equivalent MOI (3000 vg/cell) with and after 72 hours demonstrated 2-3 fold increase in transgene expression, as measured by fluorescence, in AAV2-ΔITR-up compared to AAV2-ΔITR-down ($p<0.001$). This same experiment with AAV3 demonstrated 4-5 fold increase in transgene expression for AAV3-ΔITR-up compared to AAV3-ΔITR-down ($p<0.001$), thus indicating this may be a characteristic across multiple serotype ITRs. Representative images are shown in Figure 2. This was followed up with RNA extraction and quantification by RT-qPCR, which demonstrated commensurate increase in RNA levels for ΔITR-up (AAV2 & AAV3) compared to ΔITR-down. Ultimately, this suggests transgene orientation relative the ΔITR in scAAV may not be an inert factor, at least *in vitro*, and any experiments comparing self-complementary vectors should use scAAV in the same orientation for all experimental and control groups. It is yet unknown if this effect is transient or persistent regarding long-term transgene expression, but regardless this may have clinical relevance for overall vector treatment efficiency and scAAV genome design if the superiority of scAAV-ΔITR-up over scAAV-ΔITR-down also occurs *in vivo*.

Figure 1: Self-complementary AAV Orientation

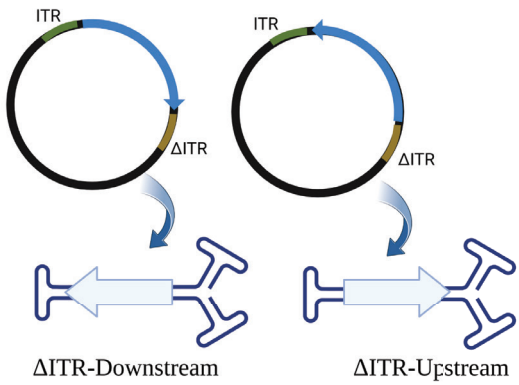
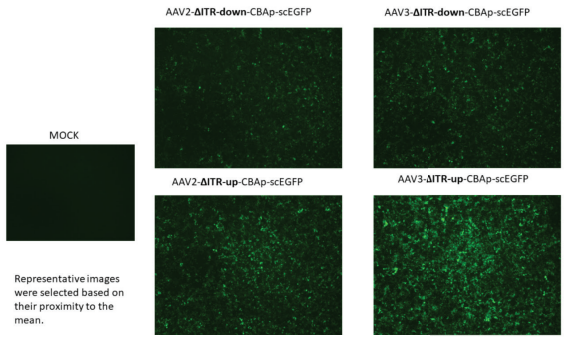


Figure 2: Representative Images



1483 Safety and Efficacy of AAV-Mediated Homology-Independent Targeted Integration at the 3 Prime Mouse Albumin Locus

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Liver-directed gene transfer with adeno-associated viral (AAV) vectors is being considered for therapy of both liver and systemic diseases. Despite their great therapeutic potential, AAV vectors are predominantly non-integrative, preventing their application to newborn liver which is the preferred target for early-onset inborn errors of metabolism.To overcome this limitation, we developed AAV-mediated homology-independent targeted integration (HITI) by CRISPR/Cas9 to integrate a therapeutic transgene at the 3' end of mouse Albumin which is highly expressed in hepatocytes. We show the efficacy of our system in a mouse model of Mucopolysaccharidosis type VI (MPSVI) lacking the lysosomal enzyme arylsulfatase B (ARSB). Neonatal AAV-HITI delivered at the highest dose tested, leads to supra-physiological and stable levels of secretion of the serum active ARSB from the liver up to 1 year of age. Our data suggest safety of AAV-HITI with unaltered levels of secreted Albumin and no evidence of hepatocellular carcinoma development at this stage. We are currently conducting off-target analysis and a dose-response study in newborn MPVI mice with doses lower than those used in the initial experiments.Overall, our data support AAV-HITI as a potential therapeutic option for the treatment of those conditions which involve the liver as target tissue and that require an early intervention.

1484 Rescuing Haploinsufficient Expression of *ADNP* in Human and Mouse Cell Lines Using CRISPR Activation

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Introduction: *Activity-dependent neuroprotective protein* (*ADNP*) Syndrome, is a rare neurological disorder caused by a pathogenic mutation in one copy of the *ADNP* gene. These mutations are *de novo* and result in a loss of function of the mutated copy of the gene causing a haploinsufficency. Clinically, patients with *ADNP*Syndrome have autism and comorbidly present with mild to severe intellectual disability, brain abnormalities (e.g., sleep disorders and seizures), organ system complications (e.g., gastrointestial and cardiovascular defects), skeletal abnormalities, and dysmorphic features such as eyelid eversion, broad nasal bridge, and thin upper lip. *ADNP* plays a critical role in brain development, cognition, and motor function. While CP201 (NAP) and ketamine have both been tested for off-label

use and/or are currently in clincial trials, methods to fully restore a healthy phenotype in these children, especially during this critical time point, has yet to be fully explored. Herein, we investigate the utility of a CRISPR activation (CRISPRa) system in an AAV-ready construct, paired with a single guide RNA (gRNA), to target and increase expression of endogenous *ADNP* in human and mouse cell lines. **Methods:** Human gRNAs, targeting the promoter of *ADNP*, were chosen from either the Gilbert, *et al*.dataset (n = 10) or designed using CHOPCHOP (n = 10). HEK293 cells were maintained and triple transfected with intein-containing vectors, each containing one terminus of dCas9 fused to either the activating domains mini-VPR (VP64-p65-Rta) or MS2-P65-HSF1 (MPH), and a gRNA. The potency of each gRNA to increase *ADNP*transcription was measured using RT-qPCR. The five most active gRNAs were then tested in a more cell and disease contextual model - human iPSCs were created carrying two common *ADNP* mutations (p.V180fs and p.Y719X) and differentiated into neural stem cells (NSCs). Concurrently, the same dCas9 system was evaluated for *Adnp* using mouse gRNAs (n = 15) in Neuro 2A cells. **Results:** 20 gRNAs were screened in the HEK293 cells, with gRNA C9 showing the greatest increase in transcription ($p < 0.0087$). The five most activating guides from the HEK293 experiments (C4, C9, C10, W5, W9) were then tested in the differentiated NSCs. C4 and W9 both showed a significant increase ($p < 0.015$ and $p < 0.0001$) in relative gene expression; 1.5- and 2-fold respectively. These guides were then multiplexed and gene expression evaluated in NSCs. Of the mouse gRNAs, C3 showed nearly a two-fold higher gene expression ($p < 0.0392$). **Conclusion and Future Directions:**A split dCas9 system with activation domains is a promising tool for altering the epigenetic state and increasing endogenous *ADNP* expression. The lead mouse guide (C3) will be packaged with the activator domains into AAV particles, which will allow for us to test the CRISPRa system *in vivo* in *ADNP* mouse models. The lead human gRNA (W9) is currently beging evaluated in engineered iPSCs differentiated into NSCs, containing common patient mutations, to evaluate rescue of cellular function.

1485 Intercellular Zinc Finger Protein Delivery for Cross-Corrective Epigenetic Regulation in the CNS

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We are developing zinc finger transcriptional regulators (ZF-TRs) to create genomic medicines for the treatment of neurological diseases. The potency, tunability, high specificity and compact size of ZF-TRs has been leveraged to successfully modulate the expression of gene targets involved in an array of neurodegenerative and neurodevelopmental disorders. However, widespread brain delivery of therapeutic transgenes remains challenging due to the limited CNS transduction efficiencies of currently available AAV capsids. Cellular secretion of ZF-TRs from AAV-transduced cells and uptake to neighboring cells could provide a new way to achieve broader ZF-TR mediated epigenetic regulation in target CNS cells by cross-correcting non-transduced cells. Here we have developed enhanced ZF-TR architectures capable of protein secretion using the secretory pathway. A secreted luciferase reporter assay using a ZF targeting an endogenous gene fused to the KRAB repressor domain (ZF-R) revealed that the addition of a signal peptide was insufficient to enable cellular secretion of ZF-R protein. However, protein engineering strategies led to >30-fold increase in the levels of secreted protein when fused to different signal peptides. Furthermore, several mutations further increased ZF-R secretion and maintained gene repression activity in donor cells. ZF-R protein secretion was demonstrated in different cell types including mouse cortical neurons and astrocytes, with >250-fold increase in the level of ZF-R secretion observed in astrocytes. Addition of a purified recombinant ZF-KRAB protein to a neuroblastoma cell line demonstrated that ZF-R proteins have intrinsic cell-penetrating ability and maintain target gene regulation activity in recipient cells. Ongoing work will focus on assays to measure cellular secretion and uptake in relevant cell types. Intercellular delivery in the CNS could significantly increase the number of cells exposed to ZF-TRs, thus increasing the potential therapeutic benefit for a range of CNS disorders.

1486 Competition of Co-Transduced CARs for Cellular Resources in Dual-Targeting CAR T Cells

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A major limitation for Chimeric Antigen Receptor (CAR) T cell therapy is tumor escape mediated by antigen loss. A possible strategy to mitigate this is to use CAR-T cells targeting multiple antigens, although the optimal strategy to develop multi-targeting CAR-T cells is yet to be defined. Here, we tested co-transduction of lentiviral vectors expressing two different CARs to generate dual-targeting CAR-T cells. One peculiarity of this strategy is that the final CAR-T cell product consists of three different CAR populations, including a double positive population expressing both CARs in the same T cell and two single-targeting populations for each CAR alone. Considering that cellular resources are limited, we hypothesized that co-expression of two CARs in the same T cell could compete to use the transcription and translational machinery. This could result in an impaired expression of one or the two CARs on the T cell surface and an imbalance in the percentages of the three populations. To verify this hypothesis, we transduced T cells with different combinations of CAR constructs, including ARI-0001, targeting CD19, and ARI-0002, targeting BCMA (both 4-1BB-based CARs developed at Hospital Clínic de Barcelona-IDIBAPS), and CD28-based CARs targeting HER2 or mesothelin. We observed that, in the majority of CAR combinations, when co-transducing two lentiviral vectors, one or both CARs were expressed at lower levels on the T cell membrane than when transduced individually. Impaired and/or imbalanced CAR expression in dual CAR-T cells was exacerbated when increasing multiplicities of infection (MOI) were used, indicating a greater competition for CAR expression as saturation of cellular machinery approached. Of note, some CARs maintained its protein expression in co-transduction regardless of the combination used and therefore competed more efficiently for limited cellular resources. CAR competition was assessed at DNA, RNA and protein level. CARs were equally integrated in the genome when they were

transduced as single CARs as compared to co-transduced with another CAR. By contrast, CAR competition showed to start at the RNA level, since lower RNA levels were detected in co-transduction when compared to single transduction, revealing that transcriptional machinery is more sensitive to gene expression burden. Finally, we demonstrated that the use of different MOIs for each co-transduced CAR or the screening of codon-optimized CAR versions are possible strategies to generate dual-targeting CAR T-cells containing balanced populations of each CAR when using the co-transduction technique. These results are of great importance for the application of dual CAR-T cells in the clinic, suggesting that the amounts of lentivirus used for the manufacturing of T cells expressing two transgenes must be carefully controlled in order to not saturate cellular resources and conserve endogenous expression intact. Our results could be applied to other strategies currently being explored to improve efficacy of CAR-T cells, for instance incorporating other therapeutic transgenes instead of a second CAR.

1487 Kinetic Analyses Reveal Very Early CpG-Specific Antagonism to AAV-Mediated Transgene Expression: Implications for TLR9 Stimulatory Effects Prior to Effector T Cell Activation Following Gene Transfer

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The success of AAV-mediated gene therapy relies on maintenance of sufficient transgene expression to provide therapeutic benefit. A significant obstacle to transgene expression are antagonistic, inflammatory innate and adaptive immune responses. CpG motif-mediated TLR9 stimulation is thought to suppress transgene expression by: (1) early (hours to days) innate activation of plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) which collude to (2) activate antigen-specific T cells (days-weeks) that extinguish transgene expression from transduced cells. Our investigations into the kinetics of transgene expression in the setting of CpG depletion led us to the present unreported finding that CpG-dependent differences in transgene expression occurred as early as 24 hours post-vector administration, a timepoint well ahead of effector T cell action. In this study, two CAG-promoter driven, anti-plasma kallikrein antibody cassettes (α-pKal) packaged in AAV9 were generated: the α-pKal transgene sequence was left either CpG retained (CpG+) or CpG depleted (CpG-). Either vector was then intravenously administered to Balb/C mice at a dose of $1x10^{13}$ vg/kg. ELISA analyses for α-pKal showed 2-3-fold increases in protein secreted into serum from mice that received CpG- vector compared to those receiving CpG+ vector at 24- and 48-hours post-vector administration. This trend in transgene expression difference continued to the study terminus (day 30). Transgene transcriptional analyses largely showed no differences in α-pKal mRNA levels in the liver, heart, and gastrocnemius muscle. Vector abundance was also measured in these tissues and results do not support observed α-pKal protein expression differences between the two groups. Immunological evaluations confirm that CpG+ vector-injected mice exhibit higher levels of very early canonical TLR9-induced inflammatory genes, including IFN-β (up to 6-fold) and CCL2 (up to 2-fold), compared to CpG- vector-injected mice. However, IL-6 exhibited a differential tissue-specific induction pattern: CpG+ vector-receiving mice exhibited up to 2-fold induction of IL-6 in the liver, but 6-7-fold reduction in other tissues. Since early TLR9 stimulation mobilizes innate immune cells, we did flow cytometry to measure various cell frequencies and repeatedly observed that a PDCA-$1^{lo/-}$CD11b^{+} CD11$c^{+/-}$ MHC-$II^{hi/lo}$ pDC-like cell population was significantly more abundant in CpG+ vector-injected mice compared to CpG-vector-injected mice. Studies are on-going to evaluate early T cell activation and effector function of the identified pDC-like cell population. In summary, TLR9-mediated antagonism of transgene expression may occur prior to effector T cell action and warrants further investigation into mechanisms that operate independently and/or support eventual T cell targeting of transduced cells.

1488 Development of an Allogeneic FAP-CAR iNKT Cell Therapy to Modulate the Immunosuppressive Stroma and Improves Anti-Tumor Immunity Against Non-Small Cell Lung Carcinoma

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Lung cancers are one of the leading causes of cancer-associated deaths worldwide in men and women, with non-small cell lung cancer (NSCLC) accounting for ~82% of diagnoses. These cancers are highly immunosuppressive and are often refractory to current immunotherapies. Given the outstanding success of CAR T cell therapies in hematological cancers, there has been a push towards generating CAR T cell therapies for other cancer types. However, CAR T cell therapies in solid tumors, such as NSCLC, have been underwhelming to date—highlighting the need for novel therapeutic approaches for patients. The lack of benefit from conventional CAR T therapy in solid tumors is mainly due to the inability to infiltrate the tumor and survive the hostile tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) in the tumor microenvironment are a major driver of resistance, enhance tumor cell proliferation and endogenous immune suppression. *Therefore, a successful CAR therapy should ideally promote i.) infiltration of CAR-expressing cells into the tumor, ii.) direct cytotoxicity against the tumor, and iii.) reshape the immunosuppressive TME*. MiNK Therapeutics is developing novel allogeneic invariant natural killer T cells (or iNKTs) to target solid tumors and TME. iNKT cells uniquely designed for tumor killing via multiple mechanisms. They can directly kill tumor cells by recognition of lipid tumor antigens and stress ligands. They can also reshape the

TME by secreting IFN-γ, which can enhance the recruitment and activation of $CD8^+$ T cells and NK cells. iNKT cells can additionally improve dendritic cell activation, selectively kill M2 suppressive macrophages, and enhance anti-tumor activity of exhausted $CD8^+$ T cells. More importantly, these cells naturally infiltrate into tissues such as the lung and liver, making them an ideal candidate for CAR therapies against solid tumors, such as NSCLC. To overcome the immunosuppression and enhance tumor control we developed a chimeric antigen receptor (CAR), which targets fibroblast activation protein (FAP)—a surface marker overexpressed on CAFs during tumor progression. MiNK-215 is an engineered iNKT adoptive cell therapy in which iNKTs express FAP-CAR scFv and secrete IL-15 to increase cell persistence. Functional characterization demonstrated that MiNK-215 specifically recognizes and kills FAP-expressing cells and promotes *in vivo* tumor control in a mouse xenograft solid tumor model. Furthermore, activated iNKT cells harboring the CAR molecule display a proinflammatory phenotype *in vitro* and *in vivo*. To address the challenge of the immunosuppression exhibited in lung tissue, we developed a NSCLC xenograft model in which orthotopic development of the tumor triggers the settlement of murine FAP-expressing CAFs. Using a mouse surrogate of MiNK-215 targeting CAFs and not the tumor, we demonstrated that the reshaping and destruction of the stroma impairs tumor progression in the lungs and improves overall survival by mediating activation and tumor infiltration of tumor-specific $CD8^+$ T cells. Taken together, we demonstrate that targeting FAP-expressing CAFs with MiNK-215 cell therapy has the capability to promote a productive therapeutic response and expand survival in solid tumor models. Furthermore, it represents an attractive off-the-shelf allogenic cell therapy in a broad range of solid tumor cancers.

1490 Application of Baculovirus Vectors in the Prevention of Gastrointestinal Diseases

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Baculoviruses are rod-shaped viruses with a circular double-stranded DNA genome of 80-180 kbp, which infect arthropods and efficiently transduce non-dividing mammalian cells *in vitro* and *in vivo*. Recombinant baculovirus virions have been used for different purposes in mammals, such as gene therapy and the development of vaccines. We investigate whether baculoviruses can be used in the prevention of gastrointestinal diseases. In humans, infection with *Helicobacter pylori* is the leading cause of gastritis, peptic ulcer, and stomach cancer (adenocarcinoma and mucosa-associated lymphoid tissue lymphoma). Despite the seriousness of the diseases, there is currently no approved *H. pylor*i vaccine. In this work, we evaluated whether a recombinant baculovirus carrying a hybrid transgene can induce an immune response against *H. pylor*i. The gene called Thp1 codes for nine epitopes of the proteins of the *H. pylori*: carbonic anhydrase, urease B subunit, gamma-glutamyl transpeptidase, Lpp20, Cag7, and CagL. The expression of the multi-epitope fusion protein was verified in HeLa cells transduced with the recombinant baculovirus Bac-Thp1. Thus, mice were inoculated by different routes with the Bac-Thp1 baculovirus. We identified a strong B cell response. Serum IgGs specific for *H. pylori* were more abundant when the Bac-Thp1 baculovirus was administered intramuscularly, while the production of specific IgAs for the bacteria in feces was higher when the vaccine was inoculated intragastrically. Noteworthy, the B cell responses lasted for several months. Baculovirus-mediated expression of antigens may be a good strategy as recombinant vaccines for various gastrointestinal diseases.

1491 Novel CGT Viral Vector Lysis Kit for AAV Enables a Standardized AAV Workflow from Cell Lysate to Absolute and Precise Quantification of Viral Titers

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Cell and gene therapies seek to target previously untreatable diseases at their source using individualized treatments. However, developing safe and effective cell and gene therapies requires strict monitoring at all stages of the development process. Adeno-associated virus (AAV) has turned into a primary modality for efficient gene therapy applications. The process of generation and purification of the viral vectors require precise quality control to enable safe and reliable dosing during clinical studies or patient care. The ability to accurately and reproducibly quantify vector titers is essential for safe and effective AAV-based gene therapies. The AAV genome is a key component for vector quantification. While several quantification assays are available, here we outline a standardized processing and quantification workflow compatible with many AAV preparations of different purities. We show that an optimized capsid lysis procedure together with an increased target accessibility allows for a robust genome titration throughout the whole viral vector manufacturing process using PCR. qPCR is a widely used method for AAV quantification due to its sensitivity and ease of use. Nevertheless, well-characterized DNA standards and assays are needed for accurate quantification. Digital PCR enables absolute quantification with unprecedented precision and a higher tolerance towards inhibitors without the need for any standards. Additionally, more than one region of interest can be quantified at once leading to further information on genome intactness. Besides AAV genome quantification, the determination of potential impurities is crucial. Manufacturers of biologics use different approaches to show, for example, low residual host cell DNA (HCD) or Mycoplasma contaminants throughout the production process and final substance. Levels of HCD must not exceed levels established by regulatory authorities, hence, HCD monitoring is an important step in the process of manufacturing since potential carryover poses a safety concern. Here we layout a dPCR-based streamlined workflow for impurity quantification with increased precision and robustness without the need for long and tedious sample preparation.

1492 AAV Compatible Recombinant MOCS1 Gene Addition Therapy Restores Molybdenum Cofactor Synthesis and Sulfite Oxidase Enzymatic Function

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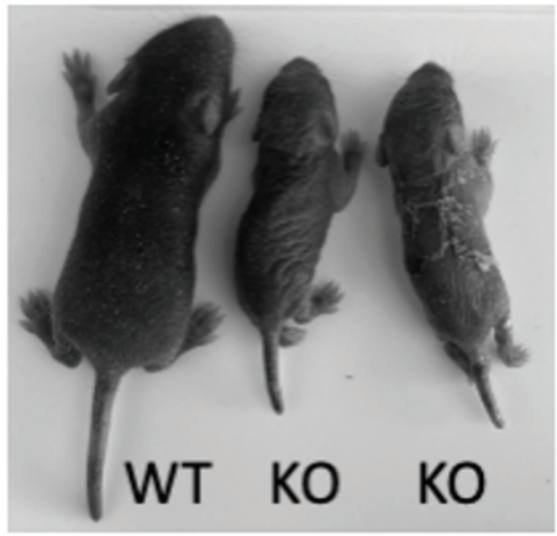
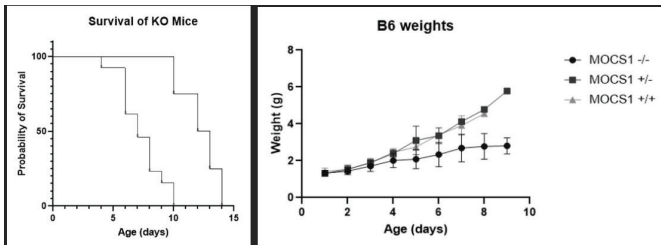
Objective: To develop a gene addition in vivo therapy for Molybdenum Cofactor type A using a construct that recapitulates the complex expression of the endogenous MOCS1 gene. Background: Molybdenum Cofactor Deficiency (MoCD) is a devastating genetic, neurodegenerative condition of the neonatal period characterized by encephalopathy, severe brain atrophy and neurological disability and early childhood death. MoCD is estimated to affect one in 200,000 newborns globally and the median survival rate is 3 years. MoCD Type A stems from mutations in the highly conserved bicistronic MOCS1 gene that produces proteins MOCS1A and MOCS1B. Loss of enzyme activity abolishes production of molybdenum cofactor and inactivates molybdenum cofactor dependent enzymes. Among these enzyme deficiencies, the loss of sulfite oxidase, which normally functions to metabolize toxic sulfite into non-toxic sulfate, is necessary and sufficient to cause the neurodegenerative phenotype of MoCD. Accordingly, the clinical, radiographic, and pathological features of MoCD are identical to those in isolated sulfite oxidase deficiency (ISOD). Current available treatments are not well tolerated or fail to prevent disease progression. Methods: We designed an optimized AAV compatible recombinant version of the endogenous MOCS1 gene to restore enzyme activity. In vitro validation of MOCS1 expression and restoration of sulfite oxidation was performed by transfection of WT and MOCS1 knockout HEK293 cells. Expression of both MOCS1A and MOCS1B was measured by western blot and downstream enzymatic activity by metabolite quantification. We compare survival, behavioral, tissue metabolism and histological characteristics of the Mocs1 knock out mouse with WT and Mocs1 heterozygote. Biodistribution and early efficacy studies were performed at 7 days following P0 retroorbital AAV-rMOCS1 injections. Results Our designed rMOCS1 construct properly expressed both MOCS1A and MOCS1B and successfully restored sulfite oxidase (SUOX) enzymatic activity in MOCS1 knockout HEK293 cells. The established Mocs1 KO mouse showed severe phenotypic differences including nephrolithiasis, hyperkeratosis , severe progressive spasticity, overall stunted growth and early death averaging at day 7. Early biodistribution studies confirmed liver expression of MOCS1A and MOCS1B 5 days after P0 injection of AAV-rMOCS1.





Conclusions Our rMOCS1 construct shows proper expression of the MOCS1 bicistronic gene and restoration of enzyme activity in vitro. The Mocs1 mouse model recapitulates survival and severe neurological phenotype seen in patients. Systemic AAV-mediated gene delivery of our rMOCS1 construct could restore sulfite oxidase deficiency and prevent neurodegeneration.

1493 Inter-Nuclear Trafficking Improves Potency of Gene Therapy Cargoes in Skeletal Muscle

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Gene editing and other DNA targeting gene therapy approaches have shown promise in treating animal models of muscle disease despite overall low editing efficiencies and poor intracellular distribution of nuclear-targeted cargos. The activity of gene therapy cargoes is influenced by the extent of spread between myonuclear domains, from transduced to non-transduced domains. Failure to spread results in accumulation of cargoes in transduced nuclei, limiting treatment effects to small regions of myofibers and reducing overall potency. Here, we developed a system using a combination of nuclear localization signals (NLS) and nuclear export signals (NES) to facilitate trafficking of gene therapy cargoes between nuclei, preventing local accumulation and improving distribution. Trafficking exposes more non-transduced nuclei to cargoes, enhancing the overall treatment effect on the myofiber. Combinations of NLSs and NESs tagged to GFP were screened in C2C12 myotubes to observe overall localization patterns and identify candidates for testing in myofibers. AAV constructs were generated that express GFP, saCas9, or dsaCas9 tagged with selected NLS/NES combinations and delivered systemically to adult wild-type and diseased mice. Myofibers were isolated from treated mice to observe localization of cargoes and treatment effects relative to transduced nuclei, and compared to the commonly used 2xSV40 NLS tag. Ai14 Cre reporter mice treated with AAV expressing Cas9 with an optimized NLS/NES combination targeted to *loxP* sites showed increased editing efficiency and improved intracellular distribution of Cas9 compared to 2xSV40 NLS-tagged Cas9. HSA^{LR} mice (a model for myotonic dystrophy type 1) treated with AAV expressing repeat-targeted dCas9 with the NLS/NES combination showed greater reduction of CUG RNA foci in regions of the myofiber farther from transduced nuclei as compared to 2xSV40 NLS-tagged dCas9. This study demonstrates that nucleo-cytoplasmic shuttling of nuclear-targeted gene therapies can increase potency by improving intracellular distribution and relieving accumulation within transduced nuclei. This strategy has the potential to improve potency of all gene editing and DNA-targeted gene therapies in skeletal muscle and other multinucleated tissues.

1494 Developing Novel Strategies to Reduce Immunogenicity of AAV Vector

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Host immune responses against viral vector present significant challenge during viral vector-based gene therapies. Novel strategies to dampen or evade host immune responses against viral vector can improve the safety and efficacy of gene therapies. Previously, we demonstrated that expression of a small immunomodulatory peptide derived from the hepatitis C virus (HCV) NS5A protein by AAV vector reduced T cell activation (TCA) and recall T cell response against AAV *in vitro*. One of the limitations of this strategy is it requires constitutive expression of immunomodulatory peptide by AAV vector, which may not be desired in all AAV vector-based gene therapy. In the current study, we sought to identity a strategy to reduce TCA via mechanism that does not require constitutive expression of immunomodulatory factor by AAV vector. Previous studies have identified a role for hepatitis B virus (HBV) e antigen (HBeAg) and the surface antigen (HBsAg) in modulating human immune responses by mechanisms that do not require intracellular expression. We hypothesized that identification of immunomodulatory motif(s) of these proteins and incorporation of these motif(s) in AAV capsid may result in immunomodulatory AAV vector. To test this hypothesis, we treated human T cells with recombinant HBeAg or HBsAg and assessed for TCA. HBeAg but not HBsAg inhibited TCA in a dose-dependent manner. To identify immunomodulatory motif(s), four 40mer peptides (P1-P4) representing entire HBeAg sequence were synthesized. Human T cells treated with peptide P1 derived from the N-terminus of HBeAg significantly inhibited TCA. Current studies are underway to further map immunomodulatory region within the HBeAg. Once immunomodulatory region is identified, we plan to incorporate HBeAg-derived immunomodulatory peptide in the AAV capsid with goal to dampen TCA during AAV gene therapy.

1495 Clinical Proof-of-Concept of AAV Gene Therapy Efficacy in Adrenomyeloneuropathy (AMN) Mice at Time of Symptoms

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Closely mimicking human AMN, Abcd1 KO mice do not show disturbed motor balance before late adulthood. Indeed, around 16-18 months, many Abcd1 KO mice shown abnormal motor balance, but no myelin lesions. We previously reported the successful prevention of neurological signs of AMN by injecting AAV9 MAG-hABCD1-HA vector intravenously (IV) into 10-d mice (Özgür Günes, HGT, 2022). These experiments opened the way for early prevention of AMN, which will only be feasible in patients who will be screened at neonatal age. On the other side, there was yet no clinical demonstration in Abcd1 KO mouse of the efficacy of gene therapy on AMN symptoms once they have already emerged. To test if gene therapy could reverse or stabilize AMN symptoms, we treated 17-month old Abcd1 KO mice with IV (1E14 vg/kg) or intra-cisterna magna (ICM) (1.5E13 vg/kg) injection of the AAV9 MAG-hABCD1-HA vector. IV injections did

not improve motor balance of the diseased mice (negligible crossing of the blood-brain-barrier by vector) and did not transduced spinal cord oligodendrocytes.In contrast, ICM injections induced an improvement of motor balance already at 3 weeks post-injection then at 3 months. **Table**: Latency to fall (seconds) at rotarod tests in vector-treated (AAV), untreated (UT), wild-type (WT) mice (mean ± sd). [a] intra-group comparison (P-value ≤0.002) at 3 weeks and 3 months versus baseline, paired t-test. [b] inter-group non-parametric comparison of AAV vs UT mice P≤0.01.

	AAV (17)	UT (8)	WT (7)
Baseline	179 ± 58	174 ± 29	259 ± 33
After 3 weeks	243 ± 50[a,b]	196 ± 44	233 ± 17
After 3 months	233 ± 52[a,b]	183 ± 38	214 ± 50

These results are currently seeking confirmation in larger groups of mice. Three months post ICM injection of vector, immunofluorescence showed abundant transduction of white matter oligodendrocytes in spinal cord at cervical and lumbar levels. Neither neurons nor astrocytes expressed the transgene. **Temporary conclusion**: AAV9 equipped with MAG promoter relieves mouse AMN axonopathy through the only transduction of spinal cord oligodendrocytes. The unexpected rapid improvement of clinical signs suggests that AMN axonopathy is still largely functional and reversible in 17 month-old mice.

1496 Benchmarking NGS Integration Site Analysis Methods in Support of Long-Term Safety Monitoring of Cell and Gene Therapy Products

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The FDA Guidance to Industry on Long Term Follow-Up (LTFU) After Administration of Human Gene Therapy Products states the importance of longitudinal testing of gene products introduced into human subjects. Depending on the delivery mechanism, the therapeutic gene product may or may not integrate into the genome. Of particular interest are gene-product integrations near proto-oncogenes which might lead to malignancies. The FDA LTFU guidance states that recipients of an integrating gene therapy modality should be tracked for 15 years, while those receiving a non-integrating therapy modality should be tracked for 5 years. Therefore, advanced analytical methods are needed to identify, quantify, and track integration events across the genome. Here, we provide a comprehensive evaluation of methods leveraging next-generation sequencing approaches for genome-wide analysis of lentiviral integration events. Our analysis employed well-characterized standards consisting of varying copy number and known integration sites. The approaches we characterized can be bucketed into two major groups: PCR amplification approaches and target capture-based approaches. All methods detected true positives with strong correlation to theoretical integration site dosage levels down to 1% allele frequency. Comparatively, PCR amplification-based approaches have lower data requirement per sample suggesting higher sensitivity, greater molecular capture, and lower limit of detection compared with target enrichment-based approaches. Target enrichment-based approaches can afford the flexibility to capture the integrated vector, which is of interest for characterizing partial integration events. While all methodologies performed well in our study, the choice of assay (or assays) for testing will depend on numerous factors including but not limited to the viral vector system and construct and starting material availability.

1497 Method for Identification and Characterization of Sites of Homology Directed Strand Cross-Over Using rAAV Integration Vectors

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Homology-directed repair (HDR) is a natural DNA repair process that maintains genome integrity through precise correction of DNA damage. Genome editing tools that utilize HDR have enormous therapeutic potential for the correction of loss-of-function disease-causing mutations. Variability in homologous recombination activity is known to exist between genome locations and DNA sequence contexts; thus identifying genomic targets with the highest likelihood of HDR is an important consideration during the development of HDR-mediated therapies. We and others have previously shown that recombinant AAVs (rAAVs) can integrate into a targeted locus by HDR without the use of a nuclease. Here, we report the application of high-throughput rAAV production and locus-wide HDR screens to identify and characterize recombinant integration at specific genomic sites that displayed increased strand cross-over. We created rAAV libraries with 0.1% - 1% base variation across the respective homology arms of >20-donor HDR rAAV plasmids. Next, we employed high-throughput rAAV packaging and *in vitro* transduction of these libraries to create an arrayed rAAV HDR screen. Long-read sequence genotyping analysis of edited alleles allowed for mapping of strand cross-over sites. The accumulated results of strand cross-over exposed potential genomic hotspots of rAAV recombination. In total, these methods represent an approach with the potential to accelerate the screening for HDR sites with improved integration efficiency as well as expand existing knowledge of genome sequence characteristics that favor strand cross-over.

1498 Expanding the Toolbox of Ubiquitous Promoters for Gene Therapy

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Adeno-associated viruses (AAVs) are a powerful tool in the field of gene therapy for delivering a therapeutic expression cassette. However, AAVs come with unique challenges, especially their limited packaging capacity of approximately < 5kb. Recombinant AAV expression

cassettes must comprise not only a therapeutic transgene, but also regulatory elements that may include a promoter and/or enhancer, an intron, and a polyadenylation signal to maximally enable gene transcription and mRNA expression, as well as ITR sequences. While many commonly-used promoters, such as CAG, often mediate strong ubiquitous expression, these promoters tend to exceed 1kb in length. Promoter size is a significant obstacle for gene augmentation when the therapeutic transgene is greater than or equal to 4kb in length. Efforts to miniaturize ubiquitous promoters have not yet yielded compact sequences with similar potency to CAG. Here, we evaluated the activity levels of several candidate ubiquitous promoters through reporter gene assays in a variety of cell types to select the best candidates for in vivo testing. Promoters that demonstrated strong ubiquitous expression were selected for miniaturization utilizing different promoter bashing techniques, including sequential ablation series, and targeted deletions guided by predictive models for transcription factor binding motifs. In this work, we anticipate identifying strong ubiquitous promoters amenable to miniaturization without compromising transcriptional potency. Additionally, we will map the key motifs contributing to overall promoter activity, as well as investigate whether swapping 5' UTRs or introns may further enable promoter miniaturization. Candidate ubiquitous promoters of less than or equal to 200bp in length will be paired with large payload transgenes (4-4.5kb in length) for assessment of in vivo function in the context of AAVs. The long-term goal will be to test these promoters for their ability to support therapeutically relevant levels of expression of large transgenes, as well as profile their degree of ubiquitous expression in heterogenous tissues, such as the eye and brain, by means of single-cell RNA-seq techniques.

1499 Understanding the Factors That Influence Capsid-Column Affinity and Peak Profile in AEX-HPLC to Measure Empty:Full Ratio

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Anion exchange chromatography by HPLC is an analytical technique that can be used to determine the empty and full capsid content of adeno-associated viral (AAV) vector drug products. AEX columns have a positively charged resin which has a high affinity for negatively charged ions (anions). Under certain conditions, AAV capsids will bind to the column and the introduction of a salt gradient will alter the ionic strength, causing the bound empty capsids to elute first from the column shortly followed by full capsids. This order of elution of the empty and full capsid is due to their slightly different isoelectric points and affinity to the column. Full and empty capsid isoelectric points differ by a pI of approximately 0.2 and so baseline separation of the capsids is a challenge. In addition, the AEX method is sensitive to small changes in chemistry, sample serotype, and environmental conditions which can make developing a reproducible empty:full method difficult. Factors such as sample preparation, mobile phase components, pH, conductivity, salt concentration, and temperature all influence the binding efficiency of AAV capsids onto the column at initial injection and the elution of the empty and full capsids during the salt gradient. Data collected during the development of an AEX empty:full method demonstrates the effects of small method changes on capsid-column affinity and peak profile. AEX results are also compared to results of other orthogonal analytical techniques such as VG/VP ratio, AUC, cIEF, CryoEM, and mass photometry.

1500 Paired HSC Epitope Engineering of CD117 (Ckit) for Antibody-Mediated Autologous Hematopoietic Stem Cell Therapy Conditioning for the Potential Treatment of Hemoglobinopathies

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Busulfan and other chemotherapeutic agents are currently used for myeloablative conditioning prior to autologous hematopoietic stem cell transplant (HSCT). Conditioning with these genotoxic agents is often associated with transplant-related morbidity which may significantly limit the use of potentially curative gene therapies for hemoglobinopathies. To overcome this unmet need, we are developing a non-genotoxic conditioning approach where we pair an engineered HSC (eHSC) expressing a modified CD117 epitope with a wild type (WT) CD117-targeted mAb for selectively depleting WT, disease harboring stem cells. We have developed two ESCAPE (Engineered Stem Cell Antibody Paired Evasion) strategies comprising of an eHSC that is multiplex base-edited to contain a CD117 variant edit and a therapeutic edit that can either cause upregulation of HbF, via *HBG1/2* promoter base editing (ESCAPE-1), or directly edit the sickle allele itself by installing a naturally occurring, non-sickling hemoglobin variant Hb G-Makassar (ESCAPE-2). We previously reported that we achieved highly efficient multiplex base editing in HSCs leading to expression of the engineered CD117 epitope with >80% base editing at desired target sites. Our engineered CD117 variants retained WT CD117 biology, binding the natural ligand SCF and exhibited ligand-induced signaling and phosphorylation competency *in vitro*. Our eHSCs also exhibited similar growth and multi-lineage colony formation potential *in vitro,* when compared with WT cells. In addition, eHSCs were capable of long-term multi-lineage hematopoietic reconstitution in rodent models. In parallel, we developed a cognate mAb that exhibited high-affinity binding to WT CD117 but exhibited loss of binding to our engineered CD117 epitopes. mAb binding blocked the interaction of WT CD117 with SCF, thereby selectively suppressing survival of the unedited HSCs. *In vitro* mAb treatment resulted in apoptosis of WT HSCs and led to >85% reduction in viability, while CD117-edited eHSCs remained viable. Importantly, mAb treatment markedly depleted FACS isolated long-term WT HSCs (Lin-CD34+CD38-CD90+CD45RA-) *in vitro*. Fc engineering of the mAb was conducted to generate

versions that reduced mast cell degranulation to baseline levels. We also performed a series of studies to evaluate efficacy of our lead anti-CD117 mAb to deplete WT HSCs and enrich for edited eHSCs *in vivo*. We were able to observe dose-dependent PK properties and receptor occupancy *in vivo*. Importantly, mAb treatment of immunocompromised mice transplanted with mixtures of WT HSCs and multiplex eHSCs harboring our ESCAPE edits resulted in substantial depletion of WT HSCs and concomitant enrichment of human eHSCs, indicating that our mAb was capable of enriching CD117-edited eHSCs *in vivo*. In summary, our ESCAPE strategy may enable non-genotoxic pre-transplant conditioning for autologous cell therapies for the potential treatment of hemoglobinopathies.

1501 Therapeutic Approach of GALV-Based Retroviral Replicating Vectors in Human and Rat Glioma Models

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Retroviral replicating vectors (RRV) have shown promising results in early-phase clinical trials of suicide gene therapy. While a multi-center Phase 3 trial of this approach in recurrent high-grade glioma patients did not meet its overall endpoints, highly statistically significant survival was observed in predetermined patient subgroups as compared to matched randomized control patients on standard-of-care treatment, and clinical investigation is on-going. In the present study, we have evaluated Gibbon ape leukemia virus (GALV) as an alternative vector platform. We developed three versions of GALV-based RRV: 1. Full GALV genome-based vector (RRV-GS5), 2. GALV-env pseudotyped MLV vecor (RRV-GC3), 3. GALV-gag-pol-env pseudotyped MLV (RRV-GSC). GALV can infect human and rat cells, so we first evaluated in vitro replication kinetics and found that RRV-GSC could infect and replicate more rapidly in human and rat glioma cells as compared to RRV-GS5 and RRV-GC3. Second, we tested in vitro cytotoxicity using RRV encoding an optimized HSV thymidine kinase enzyme (TKO). RRV-GSC-TKO vector showed ≥50%-90% reduction in cell viability after exposure to Ganciclovir prodrug in the range of 0.1μM-100μM for 5 days. Third, we examined viral stability of RRV-GSC-TKO in human glioma cells by PCR. After 21 days post-infection starting at MOI=0.01, the transgene cassette was stably retained. Finally, in both humanized mouse and syngeneic rat models, RRV-GSC-TKO+Ganciclovir treatment. showed long-term survival without undesired replication in haematopoietic cells and robust tumor growth inhibition by bioluminescence imaging.Hence, this newly developed RRV-GSC-TKO vector shows promise for further translational gene therapy.

1502 Development of a Highly Efficient Modular and Multiplexed CRISPR Editing System for Multimodal Tracking of CAR-T Cells

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Intro: Translationally relevant imaging technologies that allow one to monitor the fate of chimeric antigen receptor (CAR) T cells could enable better predictions of patient response and/or side effects, as well as help evaluate CAR-T designs. Our aim was to develop a highly efficient CRISPR system for editing human T cells with multiple transgenes at loci known to improve both safety and efficacy of CAR-T cells, whilst also making them "visible" with relevant imaging modalities. T cells were edited at the *TRAC* locus to knock-in a CAR and simultaneously knock-out the endogenous T cell receptor (TCR). Human organic anion transporting polypeptide 1B3 (*OATP1B3*) for magnetic resonance imaging (MRI), human sodium iodide symporter (*NIS*) for positron emission tomography (PET) or *Akaluc* for bioluminescence imaging (BLI) were co-engineered into our CRISPR donor vectors to allow for multi-modal *in vivo* imaging of edited CAR-T cells. **Methods:** Human T cells were nucleofected with HiFi spCas9 and gRNAs targeting *TRAC* or *AAVS1* loci. Adeno-associated viral (AAV6) vectors were designed for CD19- or HER2-CAR and reporter gene integration. Editing efficiency was determined by flow cytometry and integration by PCR. CD19+ (NALM6) or HER2+ (SKOV3) cancer cells expressing BLI reporter genes were engineered for CAR-T kill assays and preclinical mouse models. Uptake of the PET tracer [18F]tetrafluoroborate was evaluated in cells expressing NIS. Uptake of the contrast agent Gd-EOB-DTPA into OATP1B3-expressing cells was determined using 3T MRI. BLI was performed on an IVIS imaging system. **Results:** Flow cytometry showed >75% editing efficiency when targeting CAR AAVs to the *TRAC* locus. CD19CAR+/TCR- T cells significantly delayed cancer progression and extended survival in NALM6 and SKOV3 mouse models (Fig. 1). Reporter gene function was confirmed in CAR-OATP1B3 cells with MRI, in CAR-NIS cells with a gamma counter and CAR-Akaluc T cells with BLI. Multiplexing with CAR-OATP1B3/NIS at the *TRAC* locus and NIS/OATP1B3-LNGFR at the *AAVS1* locus together resulted in duel edited cells. Single and dual edited CAR-T cells effectively killed CD19+ and HER2+ cells. Correct integration at *TRAC* and *AAVS1* loci was confirmed by junction PCR analysis. In preclinical mouse models, BLI showed homing of CAR-Akaluc T cells to subcutaneous cancers (Fig. 2 A, B). As few as 1 x10^6 CAR-OATP1B3 cells pre-incubated with Gd-EOB-DTPA could be detected in subcutaneous intratumoral injections with 3T MRI (Fig. 2C). **Conclusion:** Our work describes a CRISPR-Cas system for highly efficient, modular editing of T cells with a CAR and preclinical and/or clinically relevant human reporter genes. These advanced CRISPR tools should have broad utility for co-editing primary cells with therapeutic genes and reporter genes to make trackable therapeutic cells with improved efficacy and safety profiles.

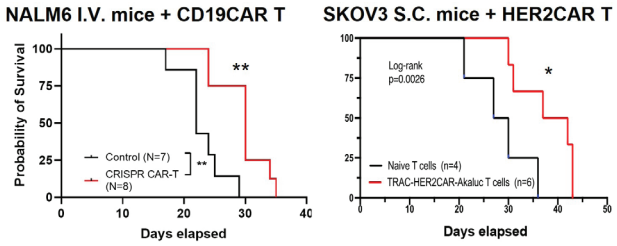

Fig.1. Significantly increased survival in NALM6 (leukemia) and SKOV3 (ovarian) cancer models treated with CD19CAR or HER2CAR T cells.

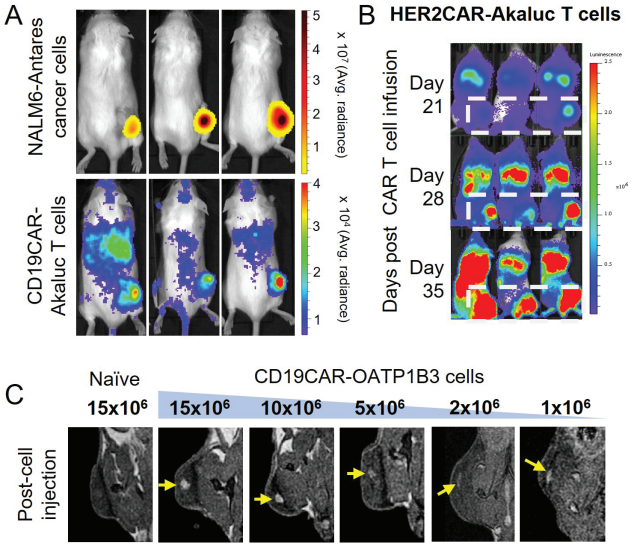

Fig.2. A) Dual BLI CAR-T cell tracking model of I.V. administered CD19CAR-Akaluc T cells homing to NALM6-Antares lesions. B) HER2CAR-Akaluc T cells homing to and expanding at SKOV3 ovarian cancer tumour lesions (dashed boxes). C) CD19CAR-OATP1B3 cells pre-incubated with Gd-EOB-DTPA showing positive contrast down to 1x10^6 cells injected intratumorally.

1503 Combining Vectorized Antibody and miRNA Lowering Strategies for Synucleinopathies

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Synucleinopathies are neurodegenerative disorders characterized by aggregation of alpha-synuclein protein (α-Syn), encoded by the SNCA (Synuclein Alpha) gene. With only some symptomatic therapies, no disease modifying treatments are available for any of the synucleinopathies. There is an immediate need to tackle the underlying pathophysiology in these diseases. Aggregated α-Syn is one of the main components of Lewy Bodies (LBs), the key neuropathological hallmarks in Parkinson's disease (PD) and Lewy Body Dementia (LBD), and of Glial Cytoplasmic Inclusions (GCI), characteristic of multiple system atrophy (MSA). The α-Syn aggregates may spread in a prion-like manner, with toxic aggregated forms being transmitted from cell-to-cell as the disease progresses. This toxic cycle can be potentially halted by decreasing the α-Syn protein levels or by facilitating the clearance of the α-Syn aggregates outside of the cell. We are developing two complementary AAV-based gene therapy approaches targeting α-Syn pathology. First, we are advancing an engineered miRNA-based approach, using our miQURE® platform, where we lower SNCA mRNA and α-Syn protein levels. We have previously shown proof-of-concept (PoC) of our SNCA-targeting miQURE approach in relevant preclinical PD models. Second, we are using our AbQURE™ platform technology for expression and secretion of vectorized, α-Syn targeting antibodies. Finally, we have combined miQURE® and AbQURE™ approaches in our GoQURE™ platform, in which we express a SNCA targeting engineered miRNA and a α-Syn targeting antibody from the same construct. Here, we have established *in vitro* PoC for AbQURE™ and GoQURE™ platforms for synucleinopathies. Two different antibodies targeting the C-terminal part of α-Syn were successfully expressed in our AbQURE™platform (AbQURE-1 and AbQURE-2). Next, these antibodies were combined with our SNCA-targeting miQURE approach in the GoQURE™ platform (GoQURE-1 and GoQURE-2). Functional antibodies were expressed in the HEK293T and SH-SY5Y cells after transfection with the plasmids carrying either the AbQURE™ or GoQURE™ constructs. Both the cell extracts and the medium of the cells were analyzed for presence of total and functional antibodies using in-house developed MSD assays. Results show that 96% of the antibodies expressed are secreted efficiently into the medium of both HEK293T and SH-SY5Y cells. The AbQURE™constructs were further packaged into AAVs and functional antibodies were successfully expressed from an AAV-AbQURE™after transduction in SH-SY5Y cells. Moreover, the SNCA-targeting miQURE encoded in the GoQURE™ constructs could also be expressed as well as the α-Syn-antibody. The present in vitro PoM results show the potential of our GoQURE™ platform to express both an engineered miRNA targeting SNCA and an α-Syn-targeting antibody from a single construct, to reduce intracellular α-Syn and target extracellular α-Syn aggregates, and ultimately decrease α-Syn toxicity in synucleinopathies. *miQURE is a registered trademark in the US and other jurisdictions; AbQURE and GoQURE are trademarks registered in the European Union and United Kingdom and pending in other jurisdictions.*

1504 Optimization of AAV Sample Preparation towards Accurate Quantification of Viral Titer by dPCR

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For AAV applications ranging from research tools to clinical-stage gene therapeutic strategies, the ability to quantify AAV vector concentration precisely and reproducibly is crucial. While several analytical methods are available, digital polymerase chain reaction (dPCR) is one of the most robust and reliable ways to determine absolute quantification of viral DNA at various stages of production. To ensure accurate titer measurements by dPCR from transgene DNA, unpurified AAV samples require DNase-I treatment to remove exogenous DNA prior to capsid disruption. Multiple factors, such as EDTA concentration and the DNase activation and inactivation method, can significantly impair the amplification efficiency of the protected viral vector genome. This highlights the potential for large discrepancies between the results of

different laboratories using analogous protocols. In this study, using a design of experiments approach, we have extrapolated an optimal DNase-I application that can be used as a standard dPCR treatment for AAVs. We determined the best concentration of EDTA to allow the inactivation of DNase-I, while not inhibiting the dPCR reaction following the treatment. Based on our findings, we have established a select protocol for processing AAV samples prior to dPCR.

1506 Efficacy of SKG0402 Gene Therapy in Fabry Disease Mouse Models

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Fabry disease is an X-linked lysosomal storage disease caused by loss-of-function mutations in the *GLA* gene encoding alpha galactosidase A (α-Gal A). Deficiency of α-Gal A leads to accumulation of globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3) in lysosomes of various cells throughout the body, resulting in a multisystemic life-threatening pathology including renal failure and cardiac disease. Current standard of care, enzyme replacement therapy (ERT), requires bi-weekly injections and has limited therapeutic effect because of poor cross-correction of renal and cardiac symptoms. To address these challenges, we developed SKG0402, an AAV9 gene therapy delivering a codon optimized human *GLA* transgene driven by a ubiquitous promoter. Intravenous administration of SKG0402 in *Gla* KO mice resulted in increased activity of human α-Gal A in a dose-dependent manner in the plasma and a variety of tissues including liver, heart and kidney. Importantly, the toxic Gb3 accumulation in these tissues was significantly decreased. Moreover, the 6-month long-term study demonstrated a sustained GLA transgene expression and durable Gb3 clearance. Additionally, we evaluated the efficacy of SKG0402 in an aggravated murine model of Fabry disease. Intravenous administration of SKG0402 in this mouse model improved survival, body weight, thermosensory and kidney functions. Severe adverse effect was not observed in all the studies performed, suggesting that the treatment with the vector was safe. Collectively, the results suggest that intravenous administration of SKG0402 may be a promising therapeutic strategy for the treatment of Fabry disease.

1507 Enhancing CRISPR Genome Editing by Extensive Chemical Modification and Self-Delivery of Guide RNAs

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Therapeutic genome editing using CRISPR-based reagents are rapidly moving to the clinic. *Ex vivo* genome editing can be potent with standard genome editing reagents (e.g. Cas9 protein or mRNA co-delivered with synthetic end-protected guide RNAs) and is currently being used successfully in clinical trials. However, therapeutic treatment of many genetic diseases will require the editing of cells and tissues directly within the patient's body. The *in vivo* activity of CRISPR reagents can be improved through partial chemical modifications of the backbone that stabilize the guide RNA from hydrolysis by endogenous nucleases (e.g. Finn, J. D. et al. *Cell Reports* 22, 2227-2235 (2018)). However, partially protected RNAs have a short half-life *in vivo* unless protected (e.g. via lipid nanoparticles). In addition, selective targeting of many tissues remains challenging with existing delivery modalities and unmodified RNA segments can stimulate counterproductive immune responses. Our strategy to improve the safety and efficiency of genome editing *in vivo* relies on the development of fully modified (FM), conjugated guide RNAs that can be delivered in a tissue-specific fashion as RNPs with the effector Cas9 protein, or co-delivered with an AAV vector expressing the effector protein and the tracrRNA. Chemical modification of the guide RNA will increase guide stability and potency, improve biodistribution, reduce immune responses, and eliminate the need for lipid nanoparticle formulation. We previously described our first generation of heavily modified (HM) crRNAs and tracrRNAs that can support effective SpyCas9 activity in mammalian cells. We have now demonstrated that our HM crRNA/tracrRNA can support efficient editing *in vivo* when directly injected into the mouse CNS as SpyCas9 RNP. We have also extended these approaches to base editors and to additional Cas9 homologues with distinct PAM specificities. Moreover, we defined new variants of HM and FM crRNA frameworks with improved Cas9 genome editing in mammalian cells and validated these guides for self-delivery in conjunction with AAV vectors that encode Cas9 and tracrRNA. This strategy promises to be particularly useful for multiplex targeting and inactivation of the AAV episome after editing is achieved, thereby minimizing genotoxicity and immune responses that could be caused by prolonged Cas9 expression.

1508 Fine-Tuning of the pH-Responsiveness of Cationic Copolymer via Amino Acid and Piperazine Substitutions for Adenovirus Delivery

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Recombinant adenoviruses (Ads) and oncolytic Ads show great potential for cancer gene therapy. However, inherent properties of Ads, like coxsackie and adenovirus receptor (CAR)-dependent endocytosis, limit their use in clinical environment where heterogenic tumors express variable expression level of CAR. To address this limitation, a pH- and glutathione-responsive poly(ethylene glycol)-poly(β-aminoester)-polyethyleneimine (PPA) was developed for conjugation with Ad. The pH sensitivity of the PPA copolymer was elegantly tuned by comparatively evaluating different amino acids (arginine, histidine, and tryptophan), piperazines (Pip1, Pip2, and Pip3), and guanidine residues in the backbone of the PPA polymer. PPA copolymer was further functionalized with bioreducible cross-linker succinimidyl 3-(2-pyridyldithio) propionate) (SPDP) to obtain PPA-SPDP to facilitate conjugation with thiolated Ad (Ad-SH), generating PPA-conjugated Ad (PPA-Ad). Ad-SH was prepared by reacting Ad with 2-iminothiolane. The size distribution and zeta potential of PPA-Ad conjugates generated with higher Ad:polymer molar ratios resulted in polymer concentration-dependent increase in both the hydrodynamic diameter and surface charge of the conjugates. The transduction efficiency of PPA-Ad conjugates in CAR-positive cells (A549 and H460 cells) was remarkably increased at the acidic pH condition mimicking those of tumor microenvironment (pH 6.2) when compared with PPA-Ad conjugate incubated under the physiological condition (pH 7.4), demonstrating its pH-dependent enhancement in cell entry. The increase in transduction efficiency was even higher for PPA-Ad conjugates in CAR-negative cells (MDA-MB-231 and T24 cells). Collectively, these results demonstrated that biocompatible and biodegradable PPA copolymers can efficiently cover the surface of Ad and increase its transduction efficiency in a pH-dependent manner.

1509 Development of a Novel Helper Plasmid: One Step Closer to the Next Generation rAAV Vectors

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Harnessing rAAVs as viral vectors for therapeutic transgene delivery still requires improvements in yields and specificity to lower vector doses, and therefore manufacturing cost, as well as to improve patient safety. To this end, our research is focused on developing novel technologies to ensure manufacturing of high yielding rAAV particles using transient transfection, as well as enhancing viral particles quality and specificity. Here we present our state-of-the art approach to design new helper plasmids (pHelpers) with the aim of improving both the genomic titers and the infectivity (TU/mL) of the viral particle obtained from suspension cultures. We took the opportunity to exploit our proprietary DNA assembly method technology to explore the synergies of multiple genetic features modularly assembled in synthetic plasmids. Comparison of the biological activity of several versions of rationally designed pHelpers led us to identify the optimal configuration able to outperform existing helper plasmids in several bioproduction conditions. Our expertise in DNA plasmid design and assembly together with our scalable transfection solutions for rAAV manufacturing give us the potential to improve both productivity and specificity of gene therapy products.

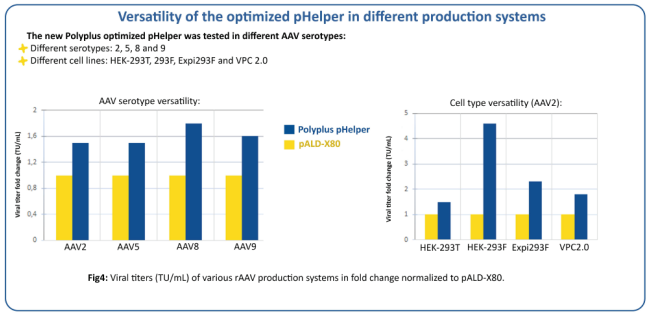

Fig4: Viral titers (TU/mL) of various rAAV production systems in fold change normalized to pALD-X80.

1510 CRISPR/Cas9 Based Genome Editing to Replace the Full-Length CFTR cDNA Shows Promising Restoration of CFTR Function and Safety in Pre-Clinical Studies

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. An autologous gene corrected airway stem cell therapy has been proposed to treat CF. The insertion of the CFTR cDNA along with a cassette expressing truncated CD19 (tCD19) in exon 1 of the CFTR gene followed by enrichment of the edited airway stem cells has been previously demonstrated to produce differentiated epithelial sheets with restored CFTR function that is comparable to non-CF controls. This Universal CFTR correction strategy (Universal strategy) can benefit almost all CF patients regardless of the causal mutation. In this study, we further characterize the safety and potency of the edited airway stem cells by characterizing the frequency of aberrant genetic rearrangements using CAST-seq, the open chromatin profile of the CFTR locus after editing using ATAC-seq and the regenerative potential of the corrected airway stem cells using single cell RNA sequencing (scRNA-seq). Using CAST-seq, translocations were observed in <0.1% of alleles and large INDELs were observed in ~1% of alleles in cells treated using the sgRNA targeting Cas9 to the ATG site of CFTR. The translocation was mapped to a known non-oncogenic off-target site that was previously reported. Human bronchial epithelial cells (HBECs) were edited using the Universal strategy and enriched to obtain populations with >70% edited cells. Upon differentiation, edited airway cells showed CFTR

function that was 127±74% relative to non-CF controls. There was no noticeable difference in the open chromatin profile of the CFTR locus between differentiated airway epithelial cells from edited and control samples apart from the region corresponding to the tCD19 cassette. Lastly, edited HBECs from each donor produced the same types of airway cells produced by control HBECs from the same donor. Analysis of differential gene expression between edited and control samples across all three donors is ongoing. Overall, our results indicate the Universal strategy does not cause adverse genomic or regulatory changes and does not limit the regenerative potential of airway stem cells.

1511 A Novel AAV RNA Based Infectivity Reporter Assay

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The infectivity of an AAV virus sample is measured by the endpoint dilution assay, which estimates the number of viruses to infect cell culture 50% of the time. The commonly used methods to estimate the median tissue culture infectious dose (TCID50) from the assay's outcome are difficult to validate, use, and quantify. Due to requirements for sample preparations, the assay is subjected to systematic error from the presence of plasmids and other nucleic acids at different points in the manufacturing process. Here we demonstrate a novel cell-based infectivity assay that measures the ability of the AAV to infect HEK293 cells. The assay uses a novel method for tagging AAV transgene's RNA and results in the expression of a fluorescent protein reporter upon transgene transcription. A 125 bp RNA tag is generated by placing a new RNA sensor module (non-coding RNA) into the post-transcriptional terminator region of the transgene. We exploit the molecular mechanism RNA pol-II transcriptional elongation beyond the terminator sequence to concurrently transcribe non-coding RNA (ncRNA) with the coding sequence of the upstream gene. As a next step, the ncRNA gets processed by the genetic machinery encoded in the engineered HEK293 cell and mediates the activation of the fluorescent reporter in a manner that reflects the activity of the gene of interest (GOI). Adding an ncRNA sequence does not involve changes to the GOI coding sequence, therefore it does not alter the packaging, regulation, or stability of GOI expression. Our data demonstrate that novel infectivity reporter assay can significantly facilitate improvements in process development workflows for both upstream and downstream optimization.

1512 Non-Viral Delivery of *ABCA4* to Photoreceptors

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Introduction: *ABCA4*-related retinopathies, including Stargardt disease, cone-rod dystrophy, and retinitis pigmentosa, are associated with mutations in the *ABCA4* gene. More than 900 mutations have been described. ABCA4 protein is localized to the outer segments of photoreceptors, and the gene encodes an importer flippase protein that prevents the accumulation of material toxic to the retinal pigment epithelium (RPE). Specifically, mutations in the *ABCA4* gene result in a build-up of lipofuscin, leading to degeneration of the RPE and subsequently, loss of photoreceptor cells. Replacement with wild type ABCA4 protein via gene therapy may slow or block disease progression. Due to the large size of the coding region (~7 kb), delivery of full length *ABCA4* is not amenable by commonly used adeno-associated virus (AAV) gene therapy platforms. Non-viral gene delivery lacks this cargo size limitation, making it an ideal approach for ABCA4 gene therapy. **Methods:** We generated covalently closed and circular DNA (C^3DNA) for codon optimized full-length human *ABCA4* and delivered it subretinally to adult porcine eyes followed by application of COMET, a proprietary electrotransfer device. **Results:** Human ABCA4 mRNA and protein expression were detected by qPCR and immunohistochemistry (IHC), respectively, at 5-6 days post-treatment. Dual labeling studies further identified the cellular and subcellular location of transgene-derived ABCA4 protein to be primarily in the outer segments of photoreceptors. Importantly, when compared to porcine ABCA4 protein, the expression pattern of human ABCA4 mimicked that of the endogenous protein. Subretinal injection requires ocular surgery that is not amenable to frequent dosing, therefore it was critical that we demonstrate long-term protein expression. We conducted a series of persistence studies in porcine retina to assess the durability of human ABCA4 protein expression. After one single subretinal injection of C^3DNA-*ABCA4*, human ABCA4 protein was readily detected in the outer segments of photoreceptors at 1, 3, 6 and 12 months, the longest time point evaluated to date. This data supports persistence of transgene-derived ABCA4 protein at least to 12 months post-treatment using Intergalactic's non-viral C^3DNA plus COMET gene delivery platform. **Conclusion:** We have demonstrated feasibility of COMET-mediated delivery of non-viral C^3DNA expressing the full length human *ABCA4* gene to relevant cell types in the retina and demonstrated the expression of human ABCA4 protein that persists at least to 12 months post-treatment in adult porcine retina.

1513 Banana Lectin H84T CARs Augment the Cytotoxic Activity of Conventional CAR T-cell Therapies to Target Multiple Solid Tumors and Their Cancer Associated Stroma

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The heterogenous and dynamic expression of tumor antigens on malignant cells and the physical barrier provided by the associated fibrotic stroma significantly hinder the success of current immunotherapies targeting solid tumors. To overcome these hurdles, we have developed a lectin-based chimeric antigen receptor (CAR) that recognizes and binds to distinct patterns of aberrant glycosylation (high mannose) specifically expressed on tumor cells and the surrounding stroma. To reduce the potential of antigen escape, we simultaneously target conventional tumor associated antigens and aberrant glycan patterns expressed on multiple types of malignant cells. We have previously demonstrated that T cells expressing a CAR with a binder

based on a modified banana-lectin (H84T BanLec) can recognize high mannose glycans expressed on pancreatic adenocarcinoma cells and tumor stellate cells and produce anti-tumor effects in both tumor spheroid and in vivo models. Since aberrant glycosylation patterns are a hallmark of cancer and are widely present on many different tumors, we hypothesized that incorporating H84T BanLec as part of a chimeric antigen receptor and expressing the construct in T cells that have been engineered to target other tumor antigens would broadly boost the efficacy of currently available immune effector cell therapies. We now show that H84T CAR expressing T-cells can effectively target and eliminate non-small cell lung cancer (NSCLC), osteosarcoma, and breast carcinoma cells both in monolayer cultures and in 3-dimensional spheroids composed of tumor cells and cancer fibroblasts. When H84T CARs are co-expressed with a conventional scFv -derived CAR targeting HER.2 we observe superior anti-tumor activity, compared to HER.2 CAR T cells alone, in the setting of NSCLC co-cultures (Figure 1) and tumor spheroid assays (Figure 2). Similarly, GD2/H84T dual CAR-T cells demonstrate greater cytotoxicity against osteosarcoma in the presence of its stromal support cells than any single CAR expressing T cell. Thus, H84T BanLec CAR T cells provide a tool to target the aberrant glycosylation patterns of malignant cells and their supporting stroma, potentially offering a broadly effective treatment option for multiple heterogenous solid tumors. We are now elucidating the underlying mechanisms by which H84T CARs induce stromal disruption and enhance tumor infiltration.

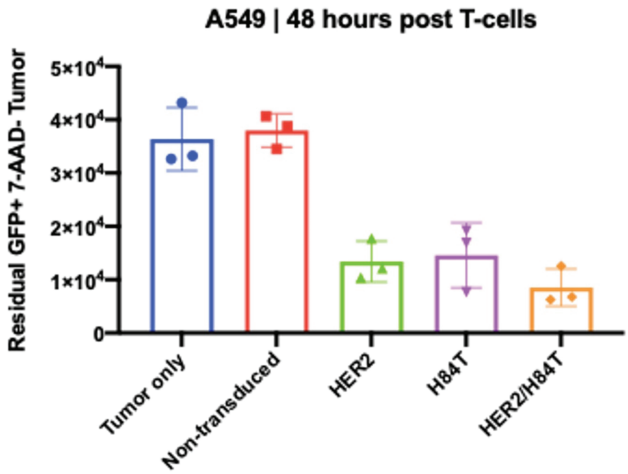

Figure 1. Dual HER2/H84T CAR T cells kill tumor better than single CARs. A549 non-small cell lung cancer co-culture data quantifying viable tumor cell count 48 hours after T cell addition. The tumor cells were transduced to express green fluorescent protein (GFP) and viable cells were selected by negative 7-AAD staining on Flow Cytometry.

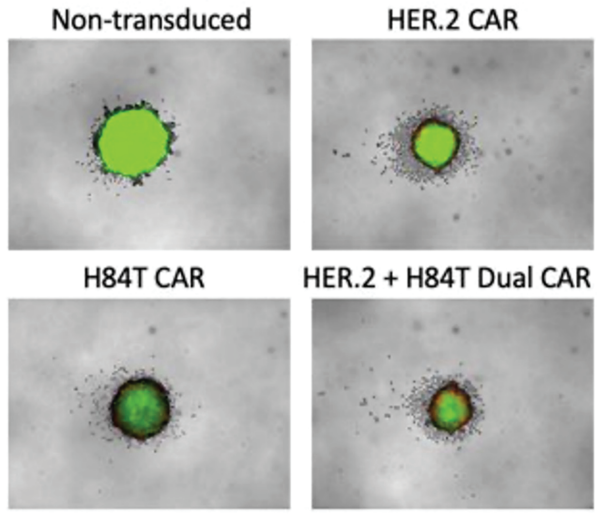

Figure 2. Dual HER2/H84T CAR T cells reduce tumor signal in 3D spheroids. A549 GFP tumor spheroid images 94 hours post T cell addition as obtained by IncuCyte Live Cell Analysis System. Annexin V-Alexa Fluor 594 conjugate was used as a marker for apoptosis and can be seen in red.

1514 Muopolysaccharidosis Type I Skeletal Alterations and Cross Correction in an *Ex Vivo* Gene Therapy Approach

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Dysostosis multiplex, one of the most prominent clinical manifestations of Mucopolysaccharidosis type I Hurler (MPSIH) patients, remains an unmet clinical need since it is not cured by the standard hematopoietic stem cell transplantation (HSCT) approach. The primary effect of the α-L-iduronidase (IDUA) enzyme deficiency is the widespread accumulation of glycosaminoglycans (GAGs) leading to a cascade of secondary events which affect cellular and extracellular functions.

The phase I/II hematopoietic stem cell-gene therapy (GT) trial (NCT03488394), involving 8 patients and currently ongoing at SR-TIGET, shows extensive metabolic correction and initial skeletal stabilization. Due to the poorly defined bone pathogenesis and bone correction after GT, we investigated the role and properties of skeletal compartment cells, i.e. mesenchymal stromal cells (MSCs), chondrocytes (CHs), osteoblasts (OBs), and osteoclasts (OCs) obtained from MPSIH patients enrolled in the trial before and after GT. The *in vitro* characterization and functionality of patient-derived MSCs and OCs were not altered by IDUA deficiency. Although MPSIH-MSCs, isolated before and after GT normally underwent the *in vitro* intramembraneous ossification process, MPSIH-OBs showed intracellular GAGs accumulation. Patient-derived gene corrected OCs, showing supraphysiologic enzyme activity levels, could cross-correct non-hematopoietic cells by releasing the IDUA enzyme. Due to the ability of MPSIH OBs to uptake the IDUA enzyme when exposed to the supernatant of patient gene-corrected OCs, mainly via mannose-6-phospate receptor (M6PR), GAG accumulation found in MPSIH-OBs was completely reversed, demonstrating the feasibility of cross-correction. Patient-derived osteomedullary biopsies (BOMs) displayed a reduced expression of type I collagen and a morphological disorganization of CHs. To deeply investigate this phenomenon, our *in vitro* 3D model of hypertrophic cartilage assessed whether patient-derived MSCs are able to generate *de novo* bone tissue after subcutaneous implantation in MPSI immunodeficient mice. In detail, the specific stainings such as Safranin, Alizarin, Picrosirius Red, together with immunohistochemistry will allow the assessment of the endochondral ossification process of MPSIH cells. Moreover, MPSIH BOMs before GT showed enthesopathy, with enlarged and vacuolated cells, that was improved and rescued in the one year post GT samples. To better characterize this feature, the enthesopathy was studied in the Achilles tendons of untreated MPSI mice, showing progressive vacuolization, matrix mineralization and calcification. Pico-CT and histomorphological analyses of MPSI mice long bones revealed a reduced number of OBs but an increased functionality, measured as bone formation rate. In addition, as early as 2 months of age, the growth plate of MPSI mice long bones showed swollen CHs and disorganization in the zone architecture. We will further study the pathogenetic mechanisms of bone damage and the skeletal correction after GT using both patient and mouse samples in order to provide a broader understanding of disease-specific mechanisms and to support clinical data.

1515 MAdCAM-1-Directed CARs Are a Novel Means of Targeting a Regulatory T Cell Therapy for Inflammatory Bowel Disease

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Introduction: Regulatory T cells (Tregs) are an attractive therapy for inflammatory bowel disease (IBD). Two major limitations in achieving widespread clinical use of Tregs are 1) the difficulties inherent in isolating and expanding pure populations of Tregs and (2), the polyclonal repertoire of Tregs, which lacks the specificity needed to maximize disease-targeted efficacy. We sought to address these challenges in the context of creating a cell therapy for IBD by directing a novel synthetic regulatory T cell platform (synRegs) to inflamed intestinal tissue via a chimeric antigen receptor (CAR) directed against the intestinal endothelial cell adhesion molecule MAdCAM-1. **Methods and Results**: Immunohistochemical analysis of intestinal samples from ulcerative colitis (UC) and Crohn's disease (CD) patients confirmed MAdCAM-1 expression in inflamed intestinal tissue and secondary lymphoid tissues. While this high level of tissue specificity makes MAdCAM-1 an attractive target for directing CAR-synRegs to the inflamed intestine, we recognized that the complex process of transendothelial migration was likely highly dependent on CAR scFV affinity that could impact migration of CAR-synRegs into the tissue. To mitigate this risk, phage panning and rabbit immunization campaigns were executed in parallel to obtain 64 human MAdCAM-1-specific antibodies with a wide range of binding affinities. These anti-MAdCAM-1 scFvs were reformatted as CARs, transduced into primary $CD3^+$ T cells using lentivirus, and screened for cell surface expression and CAR-specific T cell activation following target antigen exposure. Lead CAR candidates of varying binding epitopes and affinities were tested in an in vitro transendothelial migration assay under shear flow conditions. Critically, MAdCAM-1 CAR expression enhanced T cell binding to and migration through a $MAdCAM\text{-}1^+$ endothelial monolayer. Transmigration levels for T cells expressing certain MAdCAM-1 CARs surpassed that observed in positive control T cells expressing integrin α4β7, the natural receptor for MAdCAM-1. In vivo, human MAdCAM-1-CAR synRegs displayed preferential accumulation relative to untargeted synRegs in NSG mice bearing $MAdCAM\text{-}1^+$ tumors over the course of a two-week follow-up. Moreover, short-term homing studies also confirmed tissue entry of human MAdCAM-1-CAR synRegs into the inflamed colon of hMAdCAM-1 knock-in mice with DSS-induced colitis. **Conclusions**: MAdCAM-1 is a validated CAR target for the directing of synRegs to the inflamed intestinal tissues of IBD patients. We have identified lead binding domains that mediate antigen-specific T cell activation and promote migration across $MAdCAM\text{-}1^+$ endothelium both in vitro and in vivo, thus supporting further development of this means of targeting CAR-synRegs for the treatment of IBD.

1516 Gene Editing Proteins with Nickase Functionality Enable Scarless Targeted Gene Insertion in Primary Human Cells

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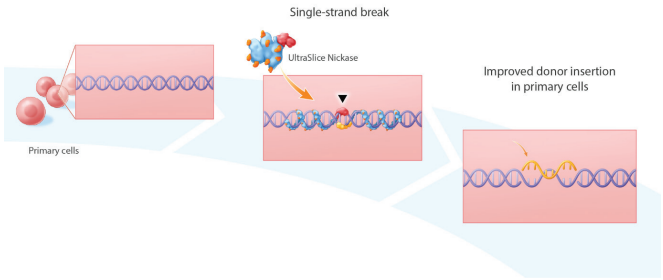
Many gene editing strategies involve the use of nucleases to generate targeted double-strand breaks (DSBs) in genomic DNA, which is often associated with cytotoxicity and off-target effects that can prevent clinical translation. Such undesirable outcomes have led to the development of gene-editing nickases, which instead create targeted single-strand breaks (SSBs) that favor high-fidelity repair through the homology-directed repair (HDR) pathway rather than the more error-prone non-homologous end joining (NHEJ) pathway. Here, we

explore the use of UltraSlice gene editing proteins containing cleavage domain variants with nickase functionality for targeted insertion of donor sequences into a defined genomic locus. Using UltraSlice gene editing proteins targeting exon 73 in *COL7A1* (mutations in which cause dystrophic epidermolysis bullosa), we tested combinations of 3 mutations previously reported to confer nickase functionality to the catalytic domain of FokI, a Type IIS restriction endonuclease (D450A, D450N, and D467A). Notably, we found that D450A and D450N resulted in significantly reduced NHEJ relative to the *COL7A1_e73* UltraSlice pair with a wild-type FokI cleavage domain, with D450N exhibiting the least amount of NHEJ. We confirmed these results with Sanger Sequencing, comparing the D450N-treated PCR amplicon to a wild-type *COL7A1* PCR amplicon and observed no significant alteration of the genomic target site. We then compared the ability of nickases to insert a 300 bp dsDNA donor sequence via electroporation into primary human fibroblasts. Insertion band intensities showed high insertion efficiencies for D450A and D450N (58.7% and 42.9%, respectively), though lower than standard UltraSlice (72.3%). Our data demonstrate that gene-editing nickases enable scarless insertion of donor sequences into defined genomic loci, and thus may have the potential to improve the safety of *in vivo* gene insertion by reducing off-target effects.



1517 Non-Viral RNA-Lipid Nanoparticles for High Efficiency Genome Editing of CD34+ Hematopoietic Stem and Progenitor Cells for Advanced Cell and Gene Therapies

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Background and Aims: The application of CRISPR-Cas9 gene editing in hematopoietic stem (HSC) and progenitor cells (HSPCs) has the potential to revolutionize the treatment of genetic blood disorders by correcting disease-causing mutations. Previously, we demonstrated the utility of a novel lipid nanoparticle (LNP) reagent for the multiplexed engineering of gene-edited CAR T cells, showing high cell viabilities, rapid onset editing, and potent CAR-mediated killing. In this current work, we demonstrate the capability of LNPs for the efficient and gentle delivery of genetic material to CD34+ HSPCs. The benefits of a non-viral LNP-mediated approach paves the way for the development of next generation gene therapy products in the HSC field. **Methods:** LNPs encapsulating S.p. Cas9 mRNA and CD33 or CD45 targeted guide RNA (sgRNA) were produced using the scalable NanoAssemblr® NxGen™ microfluidic platform technology (Fig. 1A). The LNP composition was optimized for the gentle and efficient cargo delivery to HSC/HSPCs. Purified human CD34+ cells were cultured, stimulated, and treated by the direct addition of the RNA-LNPs. Various commercial cell sources were tested including mobilized peripheral blood and cord blood. Gene knockout and viability were assessed using flow cytometry, cell proliferation using an automated cell counter, and differentiation capacity by colony-forming unit (CFU) assays. **Results:** One-step addition of LNPs to CD34+ HSPCs resulted in 84 ± 6% CD33 and 81 ± 2% CD45 knockout efficiencies (n=6 CD34+ donors, Fig. 1B). After treatment, cells maintained on average 95 ± 3% absolute cell viability, compared to 99% viability of the untreated cells, Fig. 1C. Furthermore, LNP treated HSCs maintained excellent cell proliferation, with over >90% relative cell proliferation to the untreated controls. The CFU assays showed no significant change in relative lineage formation for both the RNA-LNPs and the empty LNP vehicle control (Fig. 1D). Finally, LNP production was successfully scaled-up using microfluidics from discovery to pre-clinical scales. Conclusions: LNP-mediated CRISPR-Cas9 mRNA delivery is a promising approach for gene editing in HSPCs. The simple and gentle nature of LNP cell treatment allows for multiple genetic engineering steps for simultaneous expression and deletion of proteins for novel gene therapies. Furthermore, LNPs can be easily manufactured using microfluidics, enabling small-scale screening of RNA libraries and rapid scale-up for clinical translation.

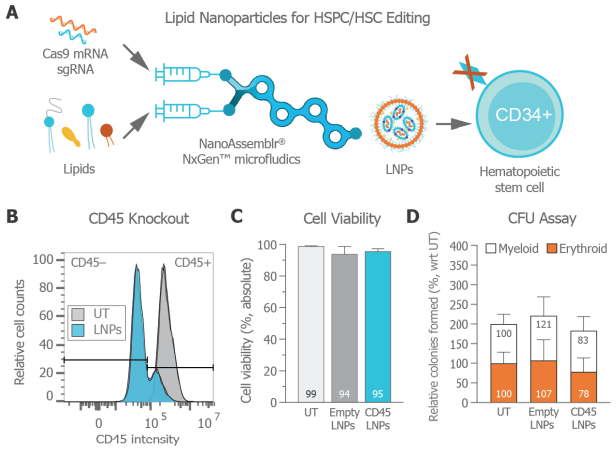

Figure 1. A) Lipids, Cas9 mRNA, and sgRNA were combined using a commercial microfluidic platform. Resulting CRISPR-LNPs were added to purified, human, CD34+ hematopoietic stem cells. **B)** CD45 CRISPR LNP treated cells show high gene knockout, as seen with flow cytometry. CD45 surface intensity rapidly diminishes, while maintained on untreated (UT) cells, one experiment shown. **C)** Absolute cell viabilities as determined with flow cytometry, UT, empty LNP and CRISPR LNP treated shown, n>4 experiments. **D)** Colony forming unit assay was conducted on untreated, empty LNP, or CD45 targeted CRISPR-LNP treated cells, n=4 unique experiments.

1518 Comparison of Cardiac Specific Promoters to Liver-Specific miRNA Targets to Maximize Cardiac *vs* Liver Expression Following Intravenous AAVrh.10-Mediated Cardiac Gene Therapy

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Intravenous (IV) administration of a cardiotropic AAV vector is the simplest and most effective strategy to deliver a gene to the myocardium for the treatment of hereditary cardiac disease. The challenge in using the IV route is that a substantial proportion of AAV vectors distribute to the liver, resulting in high liver expression with risks of liver toxicity. One approach to optimizing cardiac to liver expression levels following IV AAV administration is to modify the expression cassette to favor cardiac while minimizing liver expression. To identify the optimal strategy to enhance cardiac *vs* liver expression levels, we used the cardiotropic AAVrh.10 capsid to assess cardiac *vs* liver expression with cardiac-specific promoters *vs* the CAG highly active ubiquitous promoter driving an expression cassette with liver-specific expression-suppressing miRNA targets. Cardiac-specific promoters (TNNI3, PLN, ANKRD1, TNNT2 and MYOZ2) driving FXN were compared to expression cassettes with the CAG promoter driving FXN plus 3' liver-specific miRNA targets (miR122, 148, 192 and 194). FXN is an autosomal gene product targeted to mitochondria. GAA repeats in intron 1 of the FXN gene are responsible for Friedreich's ataxia, an autosomal recessive disorder characterized by neurologic and cardiac disease, with the cardiac disease responsible for two-thirds of deaths. Following screening *in vitro* using the HuH7 liver and A16 cardiac cells lines, cardiac-specific promoters (TNNI3, PLN, ANKRD1, TNNT2 and MYOZ2) driving FXN were compared to expression cassettes with the CAG promoter driving FXN plus 3' liver-specific miRNA targets (miR122, 148, 192 and 194). Using a human specific ELISA to assess cardiac and liver levels of human FXN protein as the outcome parameters, AAVrh.10 vectors with cardiac-specific promoters *vs* the CAG promoter with liver suppression miRNA targets were administered IV to male C57Bl/6 mice (age 6 to 7 weeks, $4x10^{12}$ gc/kg, assessed 4 weeks after administration) compared to AAVrh.10CAGhFXN as the 100% benchmark. Analysis of human FXN levels in the heart and liver of the mice 4 weeks after administration demonstrated that for the cardiac-specific promoters, the cardiac levels (compared to the CAG promoter as 100%) were 2.49% (TNNI3), 1.19% (PLN), 1.94% (ANKRD1), 9.42% (TNNT2) and 0.02% (MYOZ2). The liver FXN levels were all <0.003% of the liver expression with the CAG promoter. Thus, with the cardiac-specific promoters, there was high cardiac to liver specificity, but at the price that the cardiac levels were all <10% of cardiac levels mediated by the CAG promoter. In contrast, with the CAG promoter with liver-specific miRNA targets in the expression cassette, the cardiac levels (compared to the CAG promoter without the miRNA targets as 100%) were 27.7% (miR122), 30.1% (miR148), >100% (miR192), and 47.0% (miR194) and the liver levels were 8.6% (miR122), 26.7% (miR148), 92.8% (miR192) and 11.6% (miR194). With the highly active constitutive CAG promoter plus liver-specific miRNA targets, although the liver suppression was less than with the cardiac-specific promoter, cardiac expression was much higher. With the caveat that the data is from one transgene, one mouse strain and pending testing in larger animals, the use of a highly active constitutive promoter together with liver-specific miRNA targets, particularly miR194, enables high cardiac expression while limiting liver expression, providing an alternative to cardiac-specific promoters that may require higher doses to achieve the same cardiac expression levels.

1519 Pancreatic Cancer Gene Therapy with Liposome-Processed Midkine Gene Promoter-Introduced Oncolytic Adenovirus

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Pancreatic cancer is increasing every year and will be the second leading cause of death in 20 years. Because the number of patients with the disease is almost equal to the number of deaths, it is considered to be a representative of refractory cancers. The 5-year survival rate of pancreatic cancer is reported to be 10%, and even in the early stages, stages I-II, it is about 20-40%. Attempts have been made to improve surgical therapy, chemotherapy, radiation therapy, immunotherapy, and other therapies, but they have not improved the prognosis. Therefore, the development of novel therapeutic methods is expected. About 50 protocols of gene therapy for pancreatic cancer have been tested in clinical trials, using various viral vectors such as adenovirus, AAV, retrovirus, lentivirus, herpes virus, poxvirus, SV40 virus, and others. It is administered intratumorally, intraperitoneally, intravenously, or subcutaneously, often in combination with immunotherapy, radiation therapy, or chemotherapy, but effective results have not yet been reported. We cloned a midkine promoter with pan-tumor-specific activity and generated an oncolytic adenovirus, AdE3-midkine, transfected with this promoter. AdE3-midkine shows antitumor activity in the absence of anti-adenovirus antibody, but does not show antitumor effect in the presence of anti-adenovirus antibody due to suppression of infection. When carrier cells infected with oncolytic adenovirus are used, adenoviral infection is established even in the presence of anti-adenovirus antibody, and it shows antitumor effect, but clinical application is difficult due to severe side effects such as DIC due to acute cell disruption syndrome of carrier cells caused by the cytolytic action of oncolytic adenovirus. For this reason, we developed a new DOPE/DOTAP/EPC liposome system and processed it to coat oncolytic adenovirus, enabling oncolytic adenovirus infection even in the presence of anti-adenovirus antibodies. Mice not preimmunized with adenovirus showed no anti-tumor effect, but preimmunized mice showed 30% complete tumor regression with AdE3-midkine alone and 50% complete tumor regression with AdE3-midkine and Ad-mGM-CSF, indicating a strong anti-tumor effect by anti-adenoviral CTL induction. These results suggest that liposome-processed oncolytic adenovirus may be an effective treatment for refractory pancreatic cancer.

1520 An Efficient Approach to Check Accuracy of Viral Copy Number Assay in Droplet Digital PCR

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Viral Copy Number (VCN) assay is a critical assay in cell and gene therapy because it is used to monitor toxicity, pharmacokinetics and durability of virus modified cell therapy products. However, the assay needs to be validated for its accuracy before it can be used in clinical settings. Here, we describe an approach to check the accuracy of this assay by using a commercially available synthetic DNA called gBlocks. gBlocks are traditionally used as positive controls for quantitative PCR and Next Generation Sequencing assays. We synthesized a gBlock containing the primer and probe binding sites of Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (*WPRE*) and Ribonuclease P Protein subunit p30 (*RPP30*). The two DNA fragments are connected by Hind III endonuclease cutting site. *WPRE* is commonly included in many lentiviral vectors. *RPP30* is commonly used as a housekeeping gene in droplet digital PCR (ddPCR) because it is validated to have two copies in mammalian cells. *WPRE* and *RPP30* assays are also commonly used in ddPCR to measure viral copy number integrated in cells after lentivirus infection. Using a multiplex *WPRE/RPP30* ddPCR assay, we amplified *WPRE* and *RPP30* from the *WPRE-RPP30* gBlock with the same efficiency. Therefore, the copy number generated from *WPRE*/*RPP30* is approximately 2 regardless of input number of DNA. The copy number of *WPRE* and *RPP30* is accurately detected as low as 4.38 copies of the input gBlock number. In conclusion, gBlocks can be used as a quality control material to monitor the VCN assay and the approach can be robustly applied to other Chimeric Antigen Receptor (CAR) specific VCN assays.

1521 Structural Basis for BBB Crossing by 9P31 & 9P36 Recombinant AAVs through the Novel Receptor Carbonic Anhydrase 4

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Due to its ability to cross the blood-brain barrier (BBB), adeno-associated virus serotype 9 (AAV9) has been studied as a potential gene delivery vehicle for the treatment of brain diseases. Engineering efforts have yielded multiple brain tropism-enhanced AAV9 variants, such as PHP.eB from *Chan et al, 2017* and 9P31 and 9P36 from *Nonnenmacher et al, 2021*. All three capsids have a 7-amino-acid peptide insertion at the same position of the VR-VIII loop. PHP.eB and 9P36 also have point mutations in neighboring residues. We recently reported that 9P31 & 9P36 are distinct from PHP.eB in that they do not bind the murine BBB receptor LY6A. Instead, we identified carbonic anhydrase 4 (CA4) as a novel receptor for 9P31 and 9P36 to cross the BBB (*Shay et al, 2023*). However, the detailed molecular interactions remain unknown. Here we show by pull-down assays that CA4 selectively binds 9P31 but not PHP.eB, suggesting that CA4-mediated BBB crossing is a unique property of 9P31/9P36. We also report cryo-electron microscopy (cryo-EM) single-particle structures of 9P31 and 9P36 with resolutions of 2.24 and 2.03 Å, respectively. Both structures show more order in the C-terminal residues of the 7-mer protrusion than the N-terminal. We also solved another 2.9 Å cryo-EM structure of 9P31 in complex with mouse CA4. Unfortunately, we could not resolve CA4 electron density in this structure, but we did observe a conformational change in the 9P31 7-mer induced by CA4: the C-terminus became more ordered. Our three cryo-EM structures, together with our pull-down data and the conserved sequence of the C-terminal part of the VR-VIII loop, suggest that this C-terminal region of the 7-mer loop is responsible for recognizing and binding CA4.

1523 Knocking Down the Endogenous Bicistronic *mfrp* and *ctrp5* Transcript Benefits the Gene Therapy for MFRP Deficiency-Mediated Retinal Degeneration

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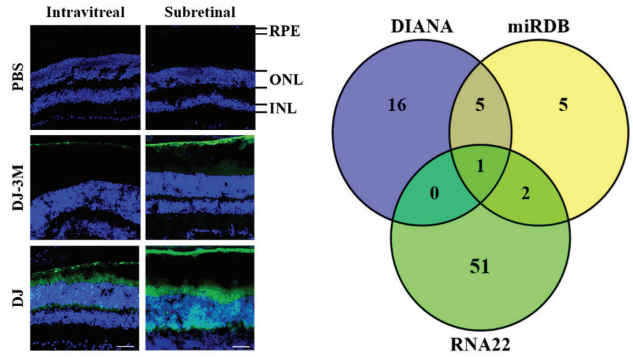
The membrane frizzled-related protein (*mfrp*) gene encodes a type II transmembrane protein predominantly in the retinal pigment epithelium (RPE) and ciliary body. A number of MFRP mutations have now been described in human, which are associated with autosomal recessive retinitis pigmentosa (RP). C1-tumor necrosis factor related protein 5 (CTRP5) is a secreted protein involving in cellular adhesion and fatty acid metabolism, whose mutation causes dominant late-onset retinal degeneration. The *mfrp* and *ctrp5* genes are expressed as a bicistronic transcript in the RPE cells. Both are associated with retinal degeneration in the eye. However, the crosstalk between these two genes and the functional role of CTRP5 in AAV-based treatment of retinitis pigmentosa that was caused by MFRP deficiency remain controversial. Here, we discovered a microRNA-based negative feedback loop to maintain normal expression level of the bicistronic *mfrp* and *ctrp5* transcript. Specifically, overexpressing MFRP significantly enhanced miR-149-3p level in the cultured cells, which in turn reduced the level of bicistronic *mfrp* and *ctrp5* transcript.First, we found the bicistronic *mfrp* and *ctrp5* transcript was significantly increased in the rd6 mice, a preclinical model of RP. We observed that when MFRP was overexpressed, there is a significant reduction in the bicistronic *mfrp* and *ctrp5* transcript both *in vitro* and *in vivo*. Next, we would like to explore the potential mechanism of MFRP's inhibitory effect on the bicistronic *mfrp* and *ctrp5* transcript. The bioinformatics analysis was conducted to predict miRNAs that may target the bicistronic *mfrp* and *ctrp5* transcript. (Fig.1) Among all the potential miRNAs, miR-149-3p was most significantly reduced in the RPE-choroid complexes of rd6 mouse eyes, compared to those of the WT mouse eyes. Overexpressing MFRP in the N2A cells significantly increased the levels of endogenous miR-149-3p. We further identified an evolutionally conserved miR-149-3p binding site in the 3'UTR of *mfrp* gene. It mediated significant decrease in the expression of upstream, cloned firefly luciferase gene upon transfection with miR-149-3p mimics *in vitro*. Thus, we concluded that MFRP inhibited the bicistronic *mfrp* and *ctrp5* transcript through miR-149-3p.More importantly, we applied our findings to AAV-based treatment of retinitis pigmentosa. We reported a capsid-modified rAAVDJ-3M vector capable of robustly and specifically transducing the RPE cells following subretinal delivery to mouse eyes. Compared to the parental vector, the modified vector induced fewer immune

responses, in terms of generating serum anti-rAAV antibodies and recruiting microglial infiltrations. (Fig.1) We observed that knocking down the bicistronic mfrp and ctrp5 transcript *in vivo* using rAAVDJ-3M-shRNA4 partially rescues rod- and cone-mediated ERG function in the rd6 mice. Furthermore, simultaneously overexpressing MFRP and knocking down the bicistronic *mfrp* and *ctrp5* transcript using rAAVDJ-3M-*mfrp*-shRNA4 provided long-term vision rescue in the *rd6* mice. Our studies provide not only new insights into the function of MFRP, but also a new therapeutic strategy for treating the MFRP-associated ocular diseases.



1524 CRISPR-Cas9-Based Gene Therapy for Treatment of IL2RA Deficiency

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Interleukin-2 (IL-2) plays a critical role in the development and function of regulatory T cells (Tregs) which are characterized by constitutive surface expression of the high-affinity IL-2 receptor alpha subunit (*IL2RA* or CD25). We have previously identified a family carrying compound heterozygous loss-of-function mutations in *IL2RA* (c.530 G>A, c.800delA) that disrupt Treg function, resulting in a range of autoimmune manifestations. One sibling in this family is more severely affected with an autoimmune pancytopenia (autoimmune hemolytic anemia, immune thrombocytopenia, and autoimmune neutropenia) requiring more aggressive clinical management. Current definitive treatment options for this patient are limited to allogeneic hematopoietic stem cell transplant (HSCT), which is associated with significant morbidity and mortality. As a potential alternative, we have developed an autologous Treg therapy providing a targeted correction of the pathogenic c.530 allele using CRISPR-Cas9 mediated homology directed repair (HDR). Here, we demonstrate an *ex vivo* manufacturing process to isolate, correct, purify, and expand *IL2RA* gene-corrected Tregs (gcTregs). Full clinical scale qualification runs were performed under current Good Manufacturing Practice (cGMP) within the UCSF Human Islet and Cellular Transplantation Facility (HICTF) with cells from a healthy donor and from the potential subject. Gene correction efficiencies were >50% following electroporation with Cas9/gRNA ribonuclear proteins (RNP) and a short DNA template encoding the wild-type IL2RA sequence. The products were sorted on IL2RA to obtain a pure population of IL2RA-corrected Tregs (>90% FOXP3$^+$, >95% IL2RA$^+$). Both qualification runs met all proposed release criteria, demonstrated Treg identity comparable to healthy donor Tregs by flow cytometry and Treg Specific Demethylated Region (TSDR) analysis, and effectively suppressed CD8$^+$ T cell proliferation in co-culture assays. In summary, we demonstrate an efficient process to manufacture gcTregs that could provide an effective treatment for patient's with the IL2RA c.530A mutation and serve as a foundation for alternative targeted gene corrections for inherited immune disorders.

1525 Role of Dendritic Cell Type 1 in AAV Mediated Antigen-Specific Immunity

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Adeno-associated virus (AAV) is a naturally occurring human/animal virus. The recombinant vectors based on these viruses have been shown to be safe for human gene therapy and generally gene transfer applications since there are no known diseases related to their infection. AAV vectors are also used for immunomodulation and the development of vaccines against infectious diseases and cancers. In order to be used for therapies that modulate the host´s immune system response and eliminate cancer cells, AAV vectors need to deliver target tumor antigens to dendritic cells (DCs). DCs are professional antigen-presenting cells located abroad the body and are critical for the activation of the adaptive and the innate immune response. There are two main populations of DCs, cDC type 1 (cDC1) and cDC type 2 (cDC2). The exact mechanism of interaction between AAV vectors with different subsets of DCs is not well understood. We previously injected capsid-optimized AAV6-S663V, expressing the green fluorescent protein in mice biceps femoris muscle and showed that these vectors target local DCs (CD11c$^+$/MHC II$^+$) that later migrate to draining lymph nodes. We further showed that AAV infection activates (CD40$^+$/CCR7$^+$) both DC1 (CD11c$^+$/MHC-II$^+$/PDCA$^-$/XCR-1$^+$/CD11b$^-$) and DC2 (CD11c$^+$/MHC-II$^+$/PDCA$^-$/XCR-1$^-$/CD11b$^+$) cells. Additionally, AAV6-S663V carrying either ovalbumin (OVA) or tyrosinase-related protein 1 (TRP1) genes merged with MHC class I molecule-trafficking signals, dramatically increases both CD4$^+$ and CD8$^+$ T cells expansion that developed 2 weeks post intramuscular administration of 1x10^{10} vg in C57BL6 mice. Results were confirmed by both flow cytometry for CD8$^+$ T cells with OVA-specific MHC I tetramers (SIINFEKL) or TRP1-specific MHC I dextramers (TAPDNLGYA) respectively, and by IFN-γ ELISPOT assays. We further observed that optimized AAV-TRP-1 delivery via intramuscular injection can elicit an immune response that is able to dramatically reduce lung metastasis by B16F10 melanoma cells spread by intravenous injection. To study the role of

cDC1 in AAV-expressing tumor antigen-based vaccination we used C57BL/6-*Irf8*^{em1Kmm} (Irf8$^{-/-}$) mice lacking cDC1 with no defects in other DC lineages. When comparing the immune response to optimized AAV-delivered TRP-1 antigen Irf8$^{-/-}$ and wild type (wt) C57BL6 mice developed a similar number of IFN-γ produced CD8^{+} T cells. Moreover, AAV-TRP-1 vaccinated Ir8$^{-/-}$ mice displayed significantly reduced lung metastasis after B16F10 melanoma cell administration with no differences compared with wt C57BL6 mice. Altogether our preliminary data suggest that cDC1 cells are not essential for the development self-antigen specific CD8^{+} T cells. However further experiments using alternative animal models, and antibody-based depletion of different effector and antigen-presenting cells are necessary to support our conclusion.

1526 The Dirtier the Better: Viral Pre-Existing Immunity a Friend of Oncolytic Viruses Based Therapy

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Oncolytic vaccines, as highly immunogenic platforms, re-gained momentum leading to public and private investments. Indeed, oncolytic viruses propagate and kill selectively cancer cells. Simultaneously, the virus infection acts as adjuvant recruiting anti-tumor immunotherapy effectors within the cancer bed. Historically oncolytic viruses have been co-developed together with their non-replicating counterpart viral vectors, that are used for gene therapy and delivery. For classical gene therapy approaches, a high level of tissue target transduction is needed hence the presence of a pre-existing anti-viral immune response, diminishes the efficacy of that vector, hampering the entire clinical protocol. On the other hand, in the field of oncolytic viruses, and specifically oncolytic immunotherapy, it is still not clear whether pre-existing immunity to the virus is deleterious or efficacious; this aspect of the oncolytic viruses needs to be elucidated. Here, we addressed the role of viral pre-existing immunity upon treatment with a platform previously developed in our lab named PeptiCRAd. This latter consists of an oncolytic adenovirus decorated with tumor MHC-I restricted peptides. First, we investigated the impact of viral neutralizing antibodies on PeptiCRAd uptake in APC (DCs and macrophages). To this end, we used SIINFEKL as a model and we pulsed primary DCs or macrophages either with PeptiCRAd-SIINFEKL or with PeptiCRAd-SIINFEKL pre-incubated with naïve (Naïve-PeptiCRAd) or adeno positive (PEI-PeptiCRAd) murine serum. In presence of PEI-PeptiCRAd-SIINFEKL, we observed an enhanced presentation of H2Kb bound SIINFEKL and CD86 in the macrophagic population, indicating that the immune adenoviral serum directed PeptiCRAd-SIINFEKL internalization toward cross-antigen presentation pathway in an immunogenic fashion. As we sought to better characterize the uptake of PEI-PeptiCRAd in macrophages, we adopted Surface Plasmon Resonance (SPR) method to analyze the intake of PEI-PeptiCRAd in RAW 264.7, a murine macrophage cell line. Confirming our previous observations, PEI-PeptiCRAd showed enhanced interaction with RAW 264.7 in comparison to Naïve-PeptiCRAd. Next, we wanted to examine the anti-tumor effect of adenoviral pre-existing immunity *in vivo* in three different immunological murine cancer models. For these studies, we selected the poor immunogenic melanoma model B16.OVA, the immunogenic colon cancer model CT26, and the immunosuppressive triple-negative breast cancer model 4T1. As mice are naïve to adenoviral infections, to generate adenoviral pre-existing immunity, we subcutaneously injected mice with adenovirus. Before engrafting the tumors, we confirmed the presence of an anti-viral immune response by the detection of anti-adenovirus IgG in the serum of pre-immunized mice and by anti-adenoviral T cell in a ELISPOT IFN-γ assay . A cohort of not-adeno preimmunized mice (naïve mice) was used as a control as well. In the poorly immunogenic B16.OVA model, the tumor growth was delayed in both Naïve and PEI mice upon PeptiCRAd treatment either using the model peptide SIINFEKL or the more clinically relevant peptide TRP2. Indeed, both flow cytometry and ELISPOT IFN-γ analysis showed the generation of antigen-specific T cells in mice treated with PeptiCRAd to the same extent in naïve and PEI mice. In contrast, in the immunogenic tumor model CT26, tumor growth control was observed in Naïve and to a better degree in PEI mice. In this latter, the presence of antigen-specific T cells was enhanced in presence of vector pre-existing immunity. Currently, 4T1 tumor model is under investigation.

1527 CRISPR Screen to Identification the Role of Tumor Microenvironment Oncometabolites on Natural Killer Cell Activity Against Solid Tumors

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Research Summary: - Background: Natural killer (NK) cells are part of the innate immune system, and possess high cytolytic activity against tumor cells, along with the ability to secrete cytokines and chemokines. However, solid tumors have been largely resistant to attack by NK cells, owing to unfavorable interactions between immune cells and tumors driven by an immunosuppressive tumor microenvironment (TME). The TME of solid tumors is characterized by elevated levels of oncometabolites. However, how their presence affects the activity of NK cells against solid tumors is not known. Among oncometabolites, we have found that 5'-Deoxy-5'-methylthioadenosine (MTA), an adenosine receptor agonist, and succinic acid, one of the metabolites of the TCA cycle, can alter NK cell behavior in the TME via different mechanisms. To understand how cancer cells respond to NK cell activity in an oncometabolite-rich environment, we studied their effects, and corresponding responses, via CRISPR screening of NK cell and solid tumor systems. - Methods and Results: NK cells were isolated from lung cancer patients and healthy adult donors. To study the effect of oncometabolites, NK cells were treated with various concentrations of oncometabolites for 24 hours, then the viability of NK cells was measured via the CCK8 assay. Lung adenocarcinoma (A549) cells were then cocultured with oncometabolite-treated NK cells in the absence or presence of oncometabolites to measure the cytotoxicity and IFN-γ production.Separately, oncometabolite-treated NK cells were used in a co-culture setting to identify drivers of the response of cancer cells to NK-mediated killing via a genome-based CRISPR screen. A549 cells were transduced with the CRISPR pooled

library and positively selected with puromycin. After co-culture with NK cells, the DNA genome was isolated and sequenced. - Conclusions: Our data showed that metabolites affect NK cell-killing capacity. Interestingly, we found that oncometabolites heterogeneously modulate the killing ability of NK cells, suggesting that contexts- and receptor-specific activation - such as the continued presence of cytokines and metabolites - drive NK cell responses to metabolic factors in the TME. Among the tested oncometabolites, MTA could drastically decrease IFN-γ levels while increasing NK cell cytotoxicity, without affecting the viability. Conversely, succinic acid showed significantly detrimental effects on NK cell viability while increasing their killing ability. We also identified genetic markers of the response of cancer cells to killing by NK cells in the presence of oncometabolites. Taken together, our data show divergent and heterogeneous effects of oncometabolites present in the TME and suggest possible genetic targets that can promote NK cell activation in these contexts.

1528 Humanized Chimeric AAV Antibodies for the Detection of Intact AAV Capsids

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Recombinant adeno-associated virus (AAV) vectors have become leading tools for viral gene therapy. Alongside the many advantages, like its apathogenic nature, there are some concerns about the safety and efficiency of AAV-based gene delivery into human cells. Depending on the AAV serotype, many humans in the general population have developed antibodies against AAV as a result of naturally acquired infections. Thus, serum antibodies against many of the wild-type AAV capsid proteins can be found in a significant proportion of the population. Intriguingly, pre-existing immunity to AAV might affect efficiency and safety of the gene transfer using AAV vectors. While the use of a high dose of AAV particles might lead to unwanted immune reactions that might put the patient at risk, the use of a low dose might fail to elicit the desired therapeutic effect. Finding the right dose without causing severe side effects but ensuring efficiency of the therapy still is a major challenge in every therapeutic approach. It is an even greater challenge in the area of virus-based gene therapy due to the limitation of a single vector application to each patient. Therefore, testing for pre-existing AAV antibodies in patient sera remains an indispensable step for the inclusion of patients into gene therapy programs. To develop reliable and reproducible AAV serology assays for the evaluation of patient groups, positive controls are crucial. For this purpose, well-characterized sero-positive samples or purified human antibodies that are verified to bind or even neutralize the corresponding AAV serotype are valuable controls. To provide this suitable and fully characterized positive control for serological analysis of patient sera, we developed recombinant humanized chimeric AAV antibodies against a number of different AAV serotypes, including AAV1, 2, 3, 5, 6, 7, 8, 9, rh10, and rh74. Our new recombinant humanized chimeric AAV antibodies have been developed based on our exclusive portfolio of neutralizing anti-AAV mouse monoclonal antibodies. The antibodies have identical sequences to our conventional mouse AAV antibodies (e.g. ADK1a, A20, ADK5b, ADK8, ADK9), but contain a humanized Fc region to allow comparability with antibodies from patient sera. Thus, these antibodies can be detected in AAV serology assays based on an anti-human secondary antibody system. Here, we show characterization data of our new humanized chimeric AAV antibodies, including cross-reactivity, stability and neutralization. There is high similarity regarding cross-reactivity when compared with our conventional mouse monoclonal parental clones. The applications of these antibodies include dot blot analyses, neutralization assays as well as ELISA.

1529 The Effect of Cell Density on the Plasmid Utilization for the Production of Adeno-Associated Virus via the Triple-Transfection Method

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Adeno-associated virus (AAV) is one of the leading vectors in the field of gene therapy with several approved treatments currently on the market. Production of AAV via the triple-transfection method is highly flexible with the capsid and gene-of-interest (GOI) being readily exchanged, facilitating rapid development. This method, however requires large quantities of high-grade plasmid DNA. To facilitate scale up and reduce the cost-of-goods, the utilization of plasmid DNA should be increased. We have previously devolved a process using adherent HEK293T cells on microcarriers to take advantage of the higher cell specific productivity seen in adherent cells and the scalability of microcarriers. However, this process was limited in the maximum cell density achievable due to several reasons, primarily shear stress. This limitation is not present in suspension cells with cell densities in excess of 80 x 10^6 cells/mL being achieved. The increase in cell density and shift to a perfusion-based process requires the screening of a large number of conditions for transfection, necessitating a scale down model.In this study a process intensification strategy was used to increase the transfection efficiency and product titer of AAV9 in suspension HEK293T cells, with a view for continuous production. A scale down model system, based on maximum power input, was used together with pseudo-perfusion to closely mimic the conditions in a stirred tank bioreactor in continuous production. Cell specific rates of glucose, glutamine, lactate, LDH, and ammonia were measured during cultivations. The AAV9 vector with green fluorescent protein (GFP) as the GOI was produced in suspension HEK293T cells via the triple-transfection method with polyethylenimine (PEI) as the transfection reagent. The impact of key transfection parameters on transfection efficiency and AAV9 titer was evaluated using flow cytometry, cell-based assays, ELISA, and qPCR. The scale down model showed a high degree of similarity to the stirred tank bioreactor with similar growth and cell specific rates in the measured metabolites being observed. Increasing cell density while maintaining a constant cell-to-DNA ratio greatly improved transfection efficiency and AAV9 production. The results of this study show that intensification of the transfection step in AAV production will increase the efficiency to which the plasmid DNA is used, thus lowering the cost-of-goods.

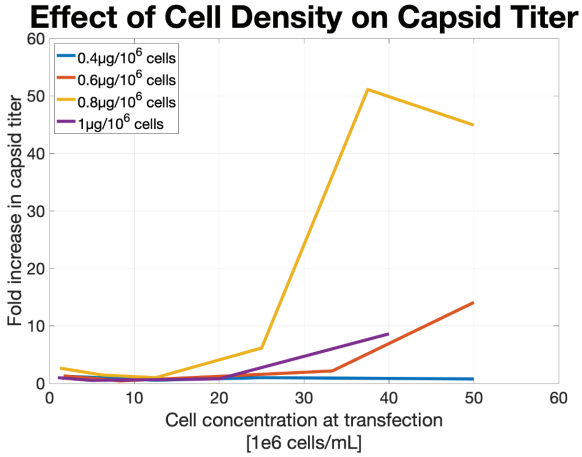

Figure 1: The effect of cell density at the time of transfection on the relative capsid titer of rAAV9 production through the triple transfection method.

1530 How Modifications of AAV Inverted-Terminal Repeats (ITRs) and *cis*-Packaging Size Impacts Vector Production

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The seminal observations that AAV2 could be rescued and replicated from proviral plasmids, following transfection into human cells, with necessary adenoviral helper functions (provided in plasmid or viral format) laid the foundation for the development of recombinant of AAV (rAAV) vectors. The discovery that the AAV ITRs were the only sequences required in *cis* for rescue, replication, and packaging of viral genomes, coupled to the observations that AAV vectors containing the AAV2 ITRs could be cross packaged with capsids from multiple AAV serotypes has simplified the use and manufacturability of AAV gene therapies using AAV2 ITRs. These GC-rich segments of DNA are 145 nucleotides long, with the first 125 bases able to form a T-shaped hairpin structure, which flanks the entire AAV genome and provides critical recognition sequences that are required for directing the AAV rep and host cell proteins to replicate and encapsidate the viral DNA into pre-assembled AAV capsids. Thus far, these short sequences of DNA have proven to be particularly problematic during vector engineering, plasmid manufacturing and scale up, predominantly due to the inherent instability of replicating these features in bacterial systems. While the use of traditional Sanger-based methods have been relatively insufficient at identifying low-level mutations within ITRs, it is only now, that newer, more sensitive, Next-Generation Sequencing technologies, have quickly been implemented as the standard quality control measure. With ever increasing scrutiny by regulatory agencies, maintaining ITR integrity is paramount from a clinical standpoint when consistent purity and potency are vital for effective gene therapies. Towards that end, we examined how ITR integrity and packaged genome sizes impacts the production of AAV vectors. Using widely established AKTA-based chromatography methods we demonstrate that undersized genomes as well as mutated ITRs significantly alter the types of virus particles produced (empty, full, partial, other). Moreover, we have developed a proprietary pCBM-ITR-containing entry vector that has intact ITRs, as well as a GC-less (<40%) tract flanking the ITR region for improved stability and overall performance. Using the triple-transfection method of AAV production, coupled to a universal two-step AKTA-based affinity and anion chromatography, we show how perturbations in ITRs impact the yields and distribution of AAV viruses produced.

1531 Developing Immunocompetent Models to Evaluate B7-H3-CAR T Cells for Acute Myeloid Leukemia

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Background: Targeting acute myeloid leukemia (AML) with chimeric antigen receptor (CAR) T cells is challenging due to high overlap of antigen expression on hematopoietic progenitor cells (HPCs) and leukemia cells and an immunosuppressive tumor microenvironment (TME). B7-H3, a coreceptor in the B7 family of immune checkpoint molecules, is an attractive target for immunotherapy as it is overexpressed in several subsets of pediatric AML (e.g., KMT2Ar, GLIS fusions) but is not found on normal HPCs. Although B7-H3-CAR T cells demonstrate anti-AML activity in xenograft models, these models lack an intact host immune system and representative AML TME required to properly assess CAR T cell anti-AML activity. To address this need, our group developed novel AML immunocompetent models where we demonstrate that mouse B7-H3 (mB7-H3)-CAR T cells are safe, with potent anti-AML activity *in vitro* and *in vivo*. **Methods/Results:** To evaluate the efficacy of mB7-H3-CAR cells for AML, we generated two immunocompetent models. In the first model we overexpressed mB7-H3 on the cell surface of AML cell line C1498 (C1498$^{mB7\text{-}H3+}$). The second model required determining natural expression of mB7-H3 on virally induced NUP98::KDM5A leukemias (N=4). We measured leukemia engraftment in C57BL/6 via bioluminescence in the C1498$^{mB7\text{-}H3+}$ model or flow cytometry in the NUP98::KDM5A model. We confirmed stable expression of mB7-H3 in the bone marrow and spleen of mice post tumor engraftment. Next, we generated mB7-H3-CAR T cells with or without co-expression of full length 41BB ligand (41BBL) by retroviral transduction of mouse splenocytes. We evaluated anti-AML activity *in vitro* using C1498$^{mB7\text{-}H3+}$ cells and noted robust, antigen-specific cytotoxicity and significant IFN-γ and IL-2 secretion elicited by mB7-H3- and 41BBL.mB7-H3-CARs ($p<0.0001$; N=4). We also show that co-expression of 41BBL allows 41BBL.mB7-H3-CAR T cells to sustain *in vitro* antitumor activity upon serial stimulation longer than mB7-H3-CAR T cells alone (N=4). To test *in vivo* antitumor activity, mice were engrafted with 1e6 NUP98:KDM5A

cells. A single of infusion of 3e6 mB7-H3-CAR T cells demonstrated significant anti-AML activity ($p<0.001$) when compared to control-CAR T cells, resulting in a survival advantage ($p<0.01$, N=5/group). To evaluate safety of mB7-H3-CAR T cells, we injected non-tumor bearing C57BL/6 mice with a single infusion of 1e7 CAR T cells (either mB7-H3- or control-CAR T cells; N=5) generated from transgenic luciferase mice. Mice were monitored via daily bioluminescence imaging for 10 days. Neither mB7-H3- nor control-CAR T cells demonstrated significant tumor-independent expansion *in vivo*. We harvested bone marrow 10 days post T cell injection. Flow cytometry analysis and colony formation assays reveal that mB7-H3-CAR T cells do not significantly impact HPCs present within the bone marrow nor their ability to proliferate and differentiate (N=3). **Conclusions:** We demonstrate that mB7-H3-CAR T cells have potent anti-AML activity *in vitro* and *in vivo,* but do not result in non-specific expansion *in vivo.* Further work will utilize our novel AML models to study how both cellular interactions and secreted factors produced within the TME impact CAR T cell functionality. These findings will inform strategies to improve CAR T cell design and bypass potential challenges faced when generating effective CAR T cells for pediatric AML.

1532 Suspension Like Scalability of AAV9 Production in Adherent Cells

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The viral vector based on the non-pathogenic, replication deficient virus, adeno-associated virus (AAV) is a very popular tool due to a favorable safety profile and little immune response. Of the four approved and five pre-registration *in-vivo* viral vector gene therapies, six use rAAV. These upcoming therapies will place a burden on current production methodologies requiring improvements in overall titers and lowering of the cost-of-goods. Production of rAAV vectors in cell aggregates could be a way to both intensify and scale up adherent cultures. Adherent cell culture is challenging to scale up, due to scaling with the surface area rather than the volume like a suspension-based process. Methods do exist to increase this scalability; such as microcarriers or fixed bed systems. These systems are however not without some limitations, namely the maximum cell density that can be achieved and the ease of use of the system. The aim of the work presented here is to circumvent these limitations by using cell aggregates. This novel rAAV allows for higher cell densities to be achieved, not only facilitating the scale up but also the intensification of AAV production. Production of rAAV9-GFP was achieved in HEK293T cell aggregates by means of the transient transfection process. It was demonstrated that this novel rAAV production method can maintain similar cell specific production of adherent cells attached to microcarriers, but in a more scalable and intensified process. Using the HEK aggregate system cell specific productivities greater than 10^4 capsids per cell have been achieved with cell densities exceeding $7 \bullet 10^6$ MVC/mL in a 200 mL bioreactor. In addition to this a simplified perfusion method was developed allowing the HEK293T aggregates to be cultured for an extended period of time, opening the door to continuous processes.

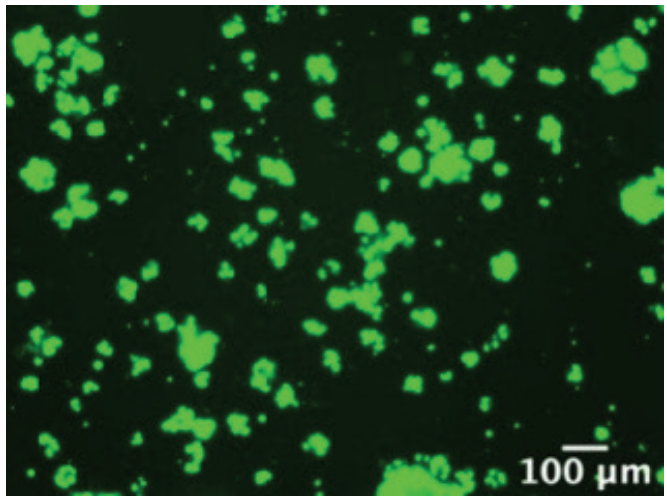

Figure 1: Fluorescent image of HEK293T cell aggregates after triple transfection for rAAV9-GFP production. Ex: 460 nm - 500 nm. Em: 512 nm - 542 nm

1533 Rescue of Hearing Loss in a STRC KO Mouse Using Multiple Dual Vector Gene Therapy Strategies

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Background Stereocilin (STRC) is a large structural protein expressed in outer hair cells (OHCs) of the cochlea, forming lateral links between adjacent stereocilia as well as attachments between the tallest row of stereocilia and the tectorial membrane. STRC is critical for OHC-mediated cochlear amplification. Human patients with loss-of-function mutations in *STRC*, classified as DFNB16, exhibit moderate hearing loss due to a defect in cochlear amplification. DFNB16 is an autosomal recessive form of hearing loss and is the second most prevalent cause of genetic auditory dysfunction in the US and EU and is a candidate for AAV-based gene replacement. Methods Due to the large size of the STRC gene and the packaging capacity of AAV, delivery of STRC gene replacement therapy to the inner ear can only be accomplished using a dual AAV approach. Using a combination of *in vitro*, *ex vivo*, and *in vivo* assays, we examined the following dual vector strategies to express STRC protein: 1) overlapping (OV), 2) dual hybrid (DH), and 3) intein trans-splicing. HEK293T cells and mouse utricle explants were transduced with dual vector tools and relative protein expression was determined via immunoblot or immunohistochemistry, respectively. For *in* vivo studies, equal titers of dual AAV were combined and delivered locally to the inner ear via the posterior semicircular canal. Immunohistochemistry was performed on cochlea whole mounts to determine levels of transgene expression. Hearing levels of untreated Strc KO mice and those treated with Strc gene therapy were assessed using auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAEs). Results

To compare the recombination efficiencies of the three dual vector strategies, we leveraged relatively high-throughput *in vitro* and *ex vivo* assays. HEK293T cells and mouse utricle explants were transduced with equal titers of each dual AAV tool (OV, DH, or intein). We found that STRC protein was produced with each dual vector strategy. Additionally, the STRC transgene was trafficked to the utricle hair cell kinocilia of STRC KO explants treated with the intein dual vector tool, demonstrating the same expression pattern seen in wild-type mice. To determine the impact of dual AAV-Strc in a Strc disease model, we delivered virus to the inner ear of neonatal Strc KO mice. Hearing (ABR and DPOAE) was improved in Strc KO mice treated with either OV or intein Strc gene therapy. Next, we quantified the number of OHCs with Strc transgene expression and found a correlation between OHC transgene expression and improvement in hearing thresholds. Lastly, we demonstrate delivery of dual vectors to the inner ear of adult mice results in efficient recombination and transgene expression in OHCs. Conclusion Our data highlights the utility of the dual vector strategies presented in delivering a large gene, such as STRC, to the inner ear of mice. The implications of these findings are important as we consider the development of gene therapy products to replace "large genes" in the inner ear.

1534 Mantarray 3D Engineered Muscle Tissue Platform Demonstrates Clinically-Relevant Disease Stratification of an *In Vitro* Duchenne Muscular Dystrophy Model

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Accurately modeling healthy and disease conditions in vitro is vital for the development of new treatment strategies and therapeutics. For cardiac and skeletal muscle diseases, direct assessment of contractile output is a reliable metric to study overall tissue function, as other 'proxy' measurements are poor predictors of muscle strength. Human 3D engineered muscle tissues (EMTs) from induced pluripotent stem cell and primary cell sources hold great potential for modeling contractile function. However, the bioengineering strategies required to generate these predictive models presents limitations for many investigators. Here, we have developed a platform that utilizes 3D EMTs in conjunction with a label-free magnetic sensing array (Mantarray). The platform enables facile and reproducible fabrication of 3D EMTs using virtually any cell source and is coupled with individual, well-based control of stimulation and highly parallel direct measurement of contractility. This approach enables clinically relevant functional measurements of muscle, stratification of healthy and diseased muscle phenotypes, and facilitates compound safety and efficacy screening. We present a 3D model of Duchenne muscular dystrophy that utilizes skeletal muscle EMTs formed from an isogenic pair of healthy and diseased cells. These tissues achieve robust twitch and tetanic responses upon stimulation. The model presents functional deficits across numerous metrics of contractility, including force and fatigability. EMTs remain functional for weeks to months in culture and provide a large experimental window to not only study therapeutic effect, but also disease phenotypes that may present at later stages of development and maturity. We have also established a method to suspend these EMTs in a biocompatible gel that permits transfer of tissues between labs. Tissues remain viable and fully functional upon gel dissolution for direct interrogation with therapeutic compounds, eliminating the need to fabricate EMTs in-house. These data demonstrate a first-and-only commercial platform integrating individual, well-based control of electrical stimulation across a 24-well plate to pace 3D tissues, modeling exercise regimens or damage protocols in muscle constructs. Stimulation is coupled with automated assessment of 3D muscle contraction, providing an inclusive, high-throughput platform for disease modeling and therapeutic discovery.

1535 Designing Cell-State-Specific Synthetic Promoters as Smart Sensors to Control Macrophage Polarization

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Background: Macrophages are a plastic cell type that can be polarized to a pro-inflammatory (M1) or an anti-inflammatory (M2) state. Macrophages localize to sites of inflammation, making them an attractive cell therapy or drug delivery vehicle, but macrophage plasticity can hamper the desired therapeutic effects. For example, M2 macrophages play a key role in promoting tissue repair in pathological conditions such as wound healing, atherosclerosis, neurodegeneration, liver fibrosis, and autoimmune disease. However, the inflammatory microenvironment converts therapeutic M2 macrophages to pathologic M1 macrophages. Here, we leverage macrophage ability to sense M1 cues to discover and engineer M1 polarization-state specific promoters that can be used to redirect endogenous signaling pathways. These promoters enable controlled immune payload expression in locoregional areas of pathological inflammation. When used to drive M2 cues, these M1 promoters enable autonomous control over macrophage polarization state to switch M1 macrophages towards an M2-like state. **Methods:** We implemented 2 strategies to discover M1 state selective promoters:1) We identified >60 genes upregulated in M1 macrophages relative to M0 and M2 macrophages across multiple transcriptomic datasets. The DNA upstream of those genes were used as native promoter sequences driving fluorescent reporters. Promoter activity was assessed by fluorescence in M1, M0, and M2 states compared to a constitutive control promoter (EFS). 2) We bioinformatically designed a synthetic promoter library containing combinatorial concatenations of M1-associated transcription factor binding motifs and enhancer sequences. Employing a massively parallel reporter assay (MPRA), we assessed promoter activity for M1, M0, and M2 macrophages across 3 biological replicates. **Results:** From approach 1, we discovered 3 promoters that had >10-fold higher activity in the M1 vs. M0 and M2 states with promoter strength at 80-240% of EFS. We optimized the weakest promoter by systematically removing regulatory elements to identify those that actively contribute to promoter activity or actively inhibit it. We then rationally re-engineered that sequence to increase promoter strength

by 3-4-fold (from 80% to 230-320% of EFS) while maintaining the same level of M1-selective activity. From approach 2, we selected >60 synthetic promoter candidates from the library with the highest-fold change in activity in the M1-state relative to the M0 and M2 states from MPRA. When clonally validated, nearly all synthetic promoters had selective activity in the M1-state, and ~14% had >10-fold selective activity in the M1 vs. M0 and M2 states. We demonstrated the utility of these cell-state-specific promoters to sense inflammatory cues and to resolve inflammation. Specifically, we used an M1 selective promoter to express an M2-associated cytokine payload, IL-10. When the engineered macrophages were exposed to M1 polarization cues, the synthetic promoter switched on to achieve state-specific expression of IL-10, which successfully suppressed M1 biomarker expression and switched the macrophages towards an M2-like phenotype. **Conclusion:** We developed and validated systematic and massively parallel strategies for discovering and engineering strong, macrophage-polarization state-selective promoters. Moreover, we demonstrated that the combination of these state-selective promoters with a cytokine payload can impose self-regulation of macrophage polarization. This work indicates that potent and state-selective promoters can be used to control cell-state and create complex "sense-and-respond" gene circuits that enable finely controlled macrophage-based cell therapies.

1536 CRISPR/Cas9 Gene Editing Increases the Risk of Tumorigenesis in Hereditary Tyrosinemia Type I

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The therapeutic potential of CRISPR gene editing has been demonstrated in various animal models, however, little is known about its long-term consequences *in vivo*. Here, we evaluate the impact of CRISPR gene therapy in an animal model of hereditary tyrosinemia type 1 (HT-1) after twelve months. CRISPR deletion of the gene hydroxyphenylpyruvate dioxygenase (*Hpd*) and pharmacological inhibition of enzyme HPD (standard of care) both demonstrate efficient metabolic correction and rescue of lethality. Surprisingly, we detect a markedly increased incidence of hepatocellular cancer in the genome-edited group, with deletions and vector integrations into the host genome. Although HT-1 is characterized by inherent cancer susceptibility, our work describes a severe adverse event associated with CRISPR gene therapy, exposing potential limitations of gene editing approaches in cancer-prone disorders.

1537 Streamlining AAV Characterization: A Capillary Electrophoresis Platform for Capsid Content Analysis

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Adeno-associated viruses (AAVs) have gained popularity in recent years for their use in therapeutic gene delivery. These complex biotherapeutics consist of both protein and genetic components, including single-stranded DNA (ssDNA) within a protein capsid. We recently published a study that analyzed the charge heterogeneity of denatured AAV capsid proteins using native fluorescence imaged cIEF, which can be used to assess identity and stability. In this study, we used the Maurice capillary electrophoresis instrument to determine if AAVs contain DNA. Our method involves analyzing two commercially available series of AAVs with varying amounts of DNA and using the results to estimate the DNA content of unknown samples.

1538 Maximizing Cationic Lipid-Based Transfection Efficiency and an Analysis of mRNA Expression in Human Prostate Cancer

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Gene therapy involves the treatment of disease by delivering new genes into a patient's cells. The mechanism of this transfer, or the vector, strongly influences the host cell response to foreign DNA. Viral gene therapy vectors are naturally equipped to deliver transgenes with precise targeting and controlled expression, however the potential risk of inducing an inflammatory immune response drives researchers to explore nonviral vectors for gene delivery. Although safer, these nonviral transfection methods are generally less efficient compared to established viral transduction. The purpose of this research is to maximize transfection efficiency of a cationic lipid-based nonviral vector (Lipofectamine LTX supplied by ThermoFisher Scientific) in human prostate cancer cells and to evaluate the host cell response to the pEF-GFP plasmid (Addgene #11154) by altering the amount of plasmid in the lipoplex formulation. The transfection efficiency was measured as the percentage of the population displaying GFP fluorescence via flow cytometry. Specifically, 0.1-2 μg of pDNA was mixed with 2.75 μL of Lipofectamine LTX and then added to wells of 100,000 PC-3 cells in a 24-well plate. The amount of plasmid DNA in the preparation had a significant effect on the transfection efficiency in PC-3 cells (see Figure 1). Surprisingly, the transfection efficiencies improved significantly at lower pDNA levels than the recommended 0.5 μg/well. We attempted to explain this phenomenon by comparing host cell gene expression patterns between PC-3 samples transfected with 0.1 μg and 1.0 μg of pDNA per well (at 48 hours post transfection). The results showed significant upregulation of a few cytokines in the samples transfected with the higher amount of pDNA (1 μg/well) compared to the low (0.1 μg/well) and non-transfected groups (see Figure 2). Two of these cytokines, IL-6 and IFNL1, are associated with activation of the JAK/STAT signaling cascade, subsequently inducing expression of several antiviral genes (AVGs) and inflammatory cytokines. The ZAP and ISG20 AVGs induce mRNA degradation and translational silencing of foreign nucleic acids. An excessive concentration of pDNA may have elicited this immune response, thereby encouraging transgene silencing

and degeneration. Altogether, these data show that inflammation can be minimized in transfected cells by minimizing the amount of pDNA delivered to the cells (while also maximizing transfection efficiency).

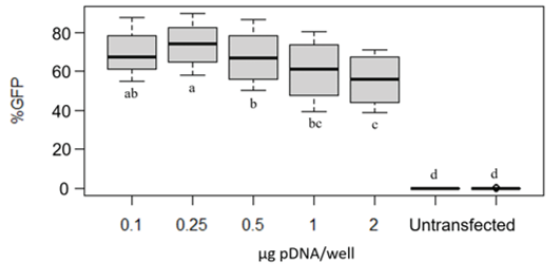

Figure 1. Box and Whisker plot for transfection efficiency in PC-3 cells with Lipofectamine over a range of pEF-GFP concentrations (0.1-2 µg/well of PC-3 cells; Untransfected groups represent negative controls that were not transfected). Cells were seeded at 50,000/well in a 24-well plate then transfected after 24 hours. Measurements of transfection efficiency (%GFP+ cells) were taken at 48 hours post-transfection. Letters (a-d) indicate groups of samples with statistically significant differences in transfection efficiency that were determined using a Friedman's test ($p < 0.05$). Error bars, or the "whiskers", extend to the maximum and minimum data points outside the upper and lower quartile range.

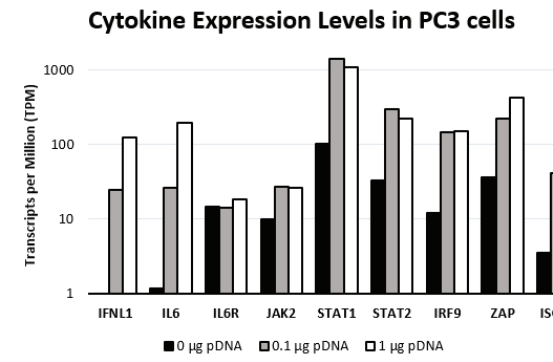

Figure 2. Bar chart showing mRNA expression levels (TPM) of significantly upregulated cytokines in PC-3 cells and the JAK/STAT pathway transfected with varying levels of pDNA (0, 0.1, and 1 µg per well). Statistical significance was determined by a Padj < 0.05 using DESeq2.

1539 CRISPNA, a New Tool for Genome Editing and Diagnosis

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CRISPR/Cas systems are powerful technologies that are changing the way scientists tackle unsolved problems in basic biology, therapy and diagnosis. The different CRISPR/Cas systems require RNA molecules (crRNAs or sgRNAs) to direct the different Cas proteins to their DNA or RNA targets. In spite of their potency and specificity, the RNA molecules could be unstable in some conditions and can allow several mismatches when binding to their target. Peptide Nucleic Acids (PNAs) artificially synthetic oligonucleotides that display higher affinity and specificity to complementary DNA and RNA compared to normal oligonucleotides. Therefore, PNA-RNA and PNA-DNA bindings are more stable and specific than RNA or DNA. In addition, their uncharged backbone makes PNAs extremely stable in biological fluids because of their resistance to proteases and nucleases. In this work, we propose the generation of a new tool called CRISPNA which combines the versatility of CRISPR-associated enzymes (Cas) with the robustness, stability, and specificity of PNAs. Our results show that PNAs can redirect different Cas proteins (Cas9 and Cas13) to their respective targets. Further analysis showed that the enzymatic activity of CRISPNA/Cas9 was abrogated while CRISPNA/Cas13 system was fully functional. We showed that the CRISPNA/Cas13 complex had sequence-specific cis- and trans-RNAase activity. Compared to CRISPR/Cas13, the CRISPNA/Cas13 complex had lower RNAase activity (near zero) in the absence of RNA target. This low background activity allows the CRISPNA/Cas13 system to outperform CRISPR/Cas13 in terms of specificity at large (3 hours) incubation times. This new development allows to open new possibilities for different applications.

1540 A Self-Amplifying RNA Vector Expressing Nanobodies Against Immune Checkpoints Induces Potent Antitumor Responses

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Immune checkpoint blockade using monoclonal antibodies (mAbs) has revolutionized the way cancer is treated. However, a large fraction of patients does not benefit from these treatments, with responses being mainly limited to immunogenic tumors. In addition, systemic administration of high doses of mAbs can generate toxicity. In the present work, we aimed to improve this therapy by optimizing both the therapeutic antibodies and their route of delivery. We generated novel nanobodies against PD-1 and PD-L1 capable of inhibiting both human and mouse interactions. Nanobodies have several advantages over conventional antibodies, such as a small size, monomeric nature, high tissue penetration and high stability. By fusing nanobodies to the fragment crystallizable (Fc) domain of mouse IgG, dimeric molecules were obtained. These dimers showed a significant reduction of IC50 for PD-1/PD-L1 interactions compared to the monomeric versions, reaching a similar or higher inhibition than commercial antibodies, including nivolumab and atezolizumab. Local administration of therapeutic antibodies could help decrease adverse systemic effects while increasing their concentration within the tumor, potentially

enhancing their therapeutic effect. To deliver nanobodies locally in tumors, we used a self-amplifying RNA (saRNA) vector based on Semliki Forest Virus (SFV). This system provides a high and transient expression of the gene of interest, inducing immunogenic cell death and type I interferon responses, which could function synergistically with immune checkpoint blockade. SFV viral particles expressing dimeric nanobodies showed a potent antitumor activity in a colorectal cancer model (MC38), resulting in >50% complete regressions and outperforming SFV vectors expressing conventional mAbs against PD-1 and PD-L1. The therapeutic potential was also validated in melanoma models (B16F10 and B16OVA). Although short-term expression of nanobodies was observed due to the cytopathic nature of the saRNA vector, it was sufficient to induce a potent inflammatory response in tumors, as observed by RNAseq and flow-cytometry analysis. Notably, increased infiltration of NK and $CD8^+$ T cells were observed in tumors treated with SFV vectors encoding nanobodies. Interestingly, the SFV system can also be employed as a non-viral vector, preserving its potential to induce cell death and inflammation due to the RNA self-amplification. Since non-viral approaches are usually easier to translate to the clinic, we tested this possibility by using a DNA plasmid able to transcribe the SFV replicon expressing the anti-PD-1 dimeric nanobody in vivo. This plasmid was administered in tumors in combination with local electroporation, yielding a strong antitumor effect in the MC38 tumor model. No indication of toxicity was observed using viral or non-viral SFV vectors encoding dimeric nanobodies. These SFV vectors constitute attractive tools that could improve the response rate to PD-1/PD-L1 inhibitors, avoiding subjecting the patients to high doses of antibodies that can have adverse effects in healthy organs. Finally, the cross-reactivity of the nanobodies described here towards human molecules is an unusual but advantageous characteristic that could pave the way for the future clinical evaluation of these agents.

1541 Therapeutic Efficacy of the Combination of Long-Term Penicillamine Treatment with a Single Administration of VTX-801 in Wilson's Disease Mice

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Wilson's disease (WD) is a rare disorder of copper metabolism inherited in an autosomal recessive manner. It is due to missense mutations in *ATP7B*, a copper-transporting P-type ATPase, that lead to hepatic, neurologic, or psychiatric symptoms. Left untreated, the condition progresses to severely debilitating complications and death. Current medical management relies on life-long oral treatment with copper chelators such as D-penicillamine (DPA) or zinc salts. VTX-801 is a recombinant adeno-associated vector (rAAV) carrying a shortened version of the human ATP7B gene. VTX-801 has demonstrated to provide long-term correction of copper metabolism and ceruloplasmin levels, preservation of liver integrity and function when administered at early stage of the disease in WD mice. Based on these results, GATEWAY, a phase I/II clinical trial was initiated to evaluate VTX-801 in adult patients with WD. We then evaluated the therapeutic effect of a single administration of VTX-801 in 20-week-old WD mice treated with DPA from 4 weeks of age. Efficacy was assessed through analysis of copper content in urine, blood and tissues, transaminases, hematology and histological analysis, including markers of inflammation and fibrosis at sacrifice, 24 weeks after VTX-801 injection. All treatments (DPA only, VTX-801 only and DPA+VTX-801) reduced the levels of hepatic copper in WD mice. Nonetheless, only the groups of mice treated with VTX-801 displayed a significantly lower concentration of copper in liver (compared to untreated WD mice), with lowest levels in mice receiving the combination. The percentage of exchangeable to total serum copper (REC), that appreciates the toxic fraction of copper in blood, was high in DPA-treated mice but was normalized by VTX-801 treatment alone or in combination with DPA. Moreover, ceruloplasmin activity in circulation was restored only in VTX-801 treated animals. These results emphasize the strong de-coppering effect of the combination therapy with long-term DPA followed by a single administration of VTX-801. Importantly, no copper deficiency was observed in those mice. Compared to the natural progression of liver pathology observed in WD mice, animals treated with VTX-801 showed a significant decrease in inflammatory cell focus/foci, diffuse hepatocyte hypertrophy and fibrosis, but also displayed increased steatosis. DPA-treated animals showed a normal liver parenchyma, when administered alone or in combination with VTX-801. When combined with DPA treatment, VTX-801 reduced severity score for all the histological parameters tested. Altogether these results indicate that treatment with a copper chelator contributes to stabilization of liver disease progression in WD mice. DPA treatment significantly improved the therapeutic efficacy of VTX-801 when administered at 20 weeks of age resulting in full restoration of copper metabolism in the absence of adverse events.

1542 Mechanistic Model for AAVs on Anion Exchange Chromatography

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AAVs are widely used delivery vehicles for gene therapy. However, the production cost of AAV-mediated gene therapy is exorbitant, necessitating targeted process development efforts. The purification of AAV capsids is particularly challenging, given capsids without transgene or an incomplete copy of the transgene can make up 70-90% of the total output from the bioreactor. The current framework for purification process development remains inefficient. Computational strategies such as mechanistic chromatography modeling based on the well-established detailed fundamental understanding of chromatography can expedite late-phase process development for empty and full capsid separation. Although modeling the transport of a large molecule, such as rAAV, through chromatography resin is very challenging, it was overcome using an advanced general rate model, which included variable surface diffusion. We demonstrate the utility of such a mechanistic model for separating a recombinant AAV capsid on Poros XQ resin. We show that the model can determine capacity, preferred loading range, conversion from gradient to step elution,

and process robustness to variation in loading material and column dimensions. Furthermore, the model's utility was demonstrated for scale-up/scale-down considerations.

1543 SKG0201: A Next Generation Gene Therapy for Spinal Muscular Atrophy with Better Efficacy and Safety Profile

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Skyline Therapeutics, Cambridge, MA

Spinal Muscular Atrophy (SMA) is caused by the homozygous deletion or mutation of the survival motor neuron 1 (*SMN1*) gene, resulting in reduced expression of SMN protein, which leads to degeneration of α motor neurons in the spinal cord and muscle atrophy. Although gene therapy product, Zolgensma, was developed and marketed, it has been reported that the drug has tolerability issues, such as significant liver toxicity, and some blood-related toxicity due to the high doses of the vector. Hence, a new AAV gene therapy for SMA with a improved safety profile and better efficacy is in desperate need. We have developed a novel AAV-mediated gene replacement product (SKG0201) which innovatively comprises an optimized human *SMN1* cDNA under the control of a unique human promoter. Every element in the vector genome has been meticulously optimized to fully attain restoration of SMN to achieve maximal therapeutic effect in the CNS while maintaining appropriate expression in peripheral tissues. Our preclinical data demonstrated that a single intravenous administration of SKG0201 resulted in a rapid and long-lasting correction of functional SMN levels, which led to a significant improvement in the body weight gain and extension in the lifespan of SMNΔ7 mice at low doses. Furthermore, SKG0201 displayed a much stronger potency with no liver toxicity compared to the reference vector (scAAV9-CMVen/CB-hSMN1), suggesting that a lower dose could be used in the clinical setting, further enhancing the safety profile of the vector. Moreover, SKG0201-treated SMNΔ7 mice exhibited a lasting efficacy in improving deficits of motor functions, preventing NMJ breakdown, and increasing the axon conductivity in six-month durability studies. The vector in the animals also manifested a strong anti-inflammatory and neuroprotective effect in the CNS, and anti-fibrosis and protective roles in the heart and liver. Additionally, our pilot 13-week study in neonatal cynomolgus monkeys (NHP) showed that a single intravenous administration of SKG0201 mediated a high expression level of hSMN in the CNS, suggesting that the expression of the transgenes was robust and long-term. Importantly, the vector was safe and well tolerated in the study. Currently, a 26-week toxicology and biodistribution study in NHP is on-going. In summary, our results strongly suggest that SKG0201, with a potentially superior efficacy and safety profile, represents a new generation of AAV gene therapy for SMA patients.

1544 Real World Evidence of Chimeric T Cell Therapy Safety: Analysis of the FDA Adverse Event Reporting System (FAERS) Database

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Introduction: With the growing population of patients receiving novel chimeric antigen T cell (CAR-T) therapies, the prevention, early identification, and management of the associated toxicities and long-term complications becomes critical. To identify patterns and trends in the adverse events (AEs) associated with CAR-T cells, we analyzed the adverse events reported in the Food and Drug Administration Adverse Event Reporting System (FAERS). The FAERS database contains voluntarily submitted reports of drug adverse events. Methods: FAERS quarterly reports files were downloaded in ASCII format. We limited the data to reports submitted from October 2017 (the date of FDA approval for the first CART product) to the latest FAERS submission (September 2022). Reports were merged and filtered by the drug involved, using string searching and manual curation. Reports involving axicabtagene ciloleucel, brexucabtagene autoleucel, lisocabtagene maraleucel, tisagenlecleucel, ciltacabtagene, or idecabtagene vicleucel were all included. Data included patient demographics, adverse event information, and outcomes. Data were processed to eliminate duplicated entries. Descriptive statistics were used to summarize the data. Adverse events were analyzed and compared between products and to the other approved treatments for similar indications. Analysis was performed using RStudio, version 4.2.2. Results: We retrieved a total of 10,388 reports on the beforementioned products. The majority of events occurred in North America (71%), followed by Europe (19%), and Asia (4%). The majority of reporters were physicians (45%). Death occurred in 2,383 (23%) reports and was most frequently reported in the tisagenlecleucel list (28%). Tisagenlecleucel had the highest median adverse events per report (4 [IQR 2-9]), while idecabtagene had the lowest (1 [IQR 1-2]). Idacabtagene reactions resulted in the lowest number of deaths, compared to the other groups (14.7%). Cytokine release syndrome (CRS) was the most frequently reported AE (50% of reports). CRS was reported the most in Idecabtagene (60%), brexucabtagene (59%), and axicabtagene (55%), while was reported less in ciltacabtagene (17%) and lisocabtagene (28%). Fifty-one percent of mortality reports had CRS listed. Immune-effector cell associated neurotoxicity (ICANS) was the second most commonly reported AE and was present in 38% of reports. ICANS occurred in 58% of brexucabtagene and 51% of axicabtagene reports, while was present in only 10% of ciltacabtagene reports. We noted reports on other malignancies or second primary cancers, which were listed in up to 7.6% of ciltacabtagene reports, 5.8% of lisocabtagene, 4.2% of axicabtagene, 1.9% of tisagenlecleucel reports, and in <1% of idecabtagene reports. Myelodysplastic syndromes and acute myeloid leukemias were the most frequently reported, followed by skin cancers. Study limitations: The study is limited to the data available in FAERS, which is subject to reporting bias and may not represent the entire population of patients who have received CAR-T products. The causality of the reported adverse events cannot be determined solely from the FAERS data. Conclusion: Our study provides further

post-marketing information on the safety profile of CAR-T products, which will help healthcare policymakers, healthcare professionals, and patients make informed decisions about the use of these products.

1545 Translational Programs in Gene Therapy and Somatic Cell Genome Editing: Nonhuman Primate Research Opportunities

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Clinical translation of emerging gene therapy and somatic cell genome editing strategies for the treatment of a range of human diseases requires access to nonhuman primate models. Nonhuman primates represent a critical bridge for translational applications because they can provide a path to understand human biological functions, study complex human diseases, and address the safety of new diagnostics and therapies proposed for human use across the life span. Of primary concern is ensuring safe and efficient editing, which represents the two most fundamental barriers to human clinical use. The Nonhuman Primate Testing Center for Evaluation of Somatic Cell Genome Editing Tools was established in 2019 to develop safe and efficient genome editing methods to treat patients with common or rare diseases. The Testing Center supports a range of NIH-funded projects targeting a variety of organ systems (e.g., brain/CNS, heart, skeletal muscle, lung, liver, kidney, hematopoietic system) through an efficient collaborative process, and is a Large Animal Testing Center in the NIH Somatic Cell Genome Editing (SCGE) Consortium. The SCGE Consortium includes 72 investigators, 45 projects, and 38 institutions across the U.S. focused on developing targeted systems for the delivery of new editors and improved human genome editing tools, and exploring new methods to assess unintended biological effects. A significant concern for the clinical use of gene editors is the potential for immune responses and pre-existing immunity. The Testing Center has a range of capabilities including total-body positron emission tomography (PET) imaging to identify edited cells and assess inflammation and T-cell trafficking. Detection of T-cell reactivity in blood may not accurately reflect local immunologic events at the target site that can reduce efficacy or result in toxicity. In the context of future human gene-editing trials, it will be important to understand how persistent gene expression leads to immune responses and the consequences of these responses for transduced/edited cells. The Testing Center collaborates with members of the SCGE Consortium through their NIH-funded grants and Collaborative Opportunity Fund projects to address these and related questions in nonhuman primate models from early prenatal stages to infants, juveniles, and adults. The Nonhuman Primate Testing Center for Evaluation of Somatic Cell Genome Editing Tools ensures contributions will have a sustained, powerful impact on new treatments for patients across all age groups, including the youngest patients in need.

1546 AAV Selection in the Kidney Reveals a Subset of Distal Tubule Cells at the Juxtaglomerular Apparatus That Are Susceptible to Transduction

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Monogenetic kidney diseases account for around 50% of nondiabetic chronic kidney diseases in pediatric cohorts and 30% in adult cohorts. To date, more than 60 genetic diseases are known to directly or indirectly affect kidneys. Adeno-associated virus (AAV) vectors have shown great potential in gene therapy. However, kidney is considered as a difficult organ for targeting due to its complex structure and function for filtration. Here, we applied a novel approach to discover a kidney-specific AAV vector. In this approach, we screened a new random AAV9 display peptide library in mice using a protocol developed for kidneys. We monitored the selection kinetics using an NGS-based scoring system and increased the selection pressure by separating renal compartments accordingly. By integrating the experimental and bioinformatics workflow we discovered a new AAV9 vector termed AAV9-JGA that specifically targeted in a subset of the thick ascending limb (TAL) characterized as SLC12A1/CLDN16-positive cells at juxtaglomerular apparatus (JGA) after systemic administration. Intriguingly, we demonstrated that this subset of TAL at JGA is generally susceptible to AAV transduction and revealed a nature transduction route of AAV in the kidney from the bloodstream. We suggest that the targeting specificity of AAV9-JGA is due to the anatomical features of JGA and the different characteristics of TAL cell subtypes in the kidney.In conclusion, we developed a comprehensive strategy and workflow to screen AAV library in vivo and discovered a new AAV vector termed AAV9-JGA which targets a subset of TAL at JGA. We revealed a common transduction route of AAV in the kidney after systemic administration. AAV9-JGA with the ability and potential to target and modulate JGA provides new possibilities for basic research and kidney disease-specific therapy.

1547 Autologous *Ex-Vivo* Lentiviral Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I): Interim Results from an Ongoing Phase 1/2 Study

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Background: Severe leukocyte adhesion deficiency-I (LAD-I) is an autosomal recessive inborn error of immunity due to mutations in the

ITGB2 gene that encodes the common subunit of β2-integrins (CD18), essential for neutrophil adhesion to the inflamed endothelium and transmigration into tissues. Severe LAD-I, defined as <2% of normal CD18 polymorphonucleocyte [PMN] expression, is characterized by frequent and often refractory bacterial and fungal infections, impaired wound healing, and significant pediatric mortality in the absence of allogeneic hematopoietic stem cell transplant (alloHSCT). AlloHSCT is potentially curative but limited by donor availability, graft-versus-host disease, and graft failure. The gene therapy RP-L201-0318 (NCT03812263) employs autologous CD34+ cells transduced with a lentiviral vector carrying *ITGB2* to restore CD18 expression. **Methods:** Patients ≥3 months old with severe LAD-I were enrolled. Hematopoietic stem cells (HSCs) were collected via apheresis after mobilization with granulocyte-colony stimulating factor and plerixafor and transduced *ex-vivo* with Chim-CD18-WPRE-LV. Myeloablative therapeutic drug monitoring (TDM) busulfan conditioning preceded RP-L201 infusion. Patients were followed for safety and efficacy measures, including survival to age two and ≥1-year post-infusion, peripheral blood (PB) PMN CD18 expression, PB vector copy number (VCN), neutrophilia improvement, decrease in infections/hospitalizations, and resolution of skin/periodontal abnormalities. **Results:** Nine patients (age 5 months to 9 years) received RP-L201 with follow-up of 12 to 36 months. RP-L201 cell doses ranged from $2.8x10^6$ to $10x10^6$ CD34+ cells/kg with a drug product VCN of 1.8 to 3.8. All nine patients demonstrated PMN CD18 restoration (median expression of 49.7%) with sustained, stable genetic markings (median PB mononuclear cell VCN of 1.72). The overall survival (OS) rate per Kaplan-Meier estimate is 100%, with all patients alive 1 year after RP-L201 infusion (and at age 2y for those enrolled at age ≤1y); no patients have had graft failure or required alloHSCT. Pre-treatment leukocytosis improved uniformly. Infection-related hospitalizations, including prolonged hospitalizations (≥7 days) were significantly reduced following therapy. No RP-L201-related serious adverse events (SAEs) were reported. No evidence of replication-competent lentivirus has been observed. Insertion site analyses indicate highly polyclonal integration patterns across the entire cohort. **Conclusion**: RP-L201 has a favorable safety profile and confers durable correction of the severe LAD-I phenotype in all nine pediatric patients treated, as demonstrated by follow-up of ≥ 1 year encompassing all relevant laboratory and clinical parameters.

1548 A Novel Knockout-Independent CRISPR Strategy to Treat LDLR-Mediated Heterozygous Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is a prevalent autosomal dominant disorder characterized by lifelong elevation in low-density lipoprotein cholesterol (LDL-C), which results in early-onset atherosclerosis and coronary events. The vast majority of genetically confirmed FH is caused by pathogenic mutations in the LDLR gene, haploinsufficiency of which leads to reduced LDL-C uptake. Currently available lipid lowering therapies often fail to attain desired LDL-C levels and require lifelong treatment. Furthermore, as FH patients often require higher doses of statins or combination therapy, these patients may be more prone to side effects or develop drug intolerance. We present a "one-and-done", direct and long-lasting therapeutic strategy for upregulating LDLR expression by truncating a section of the *LDLR* gene 3'UTR via CRISPR-based gene editing. For this purpose, we utilized OMNI-A2, a novel proprietary nuclease that we discovered, with high activity and specificity and small size, compatible with encapsulation in lipid nanoparticles. We used this approach in the HepG2 human liver cell line and the Hepa1-6 mouse hepatoma cell line, to assess excision efficiency, changes in LDLR levels and LDL-C uptake. Excised cells showed a significant upregulation of *LDLR* mRNA levels and increase in membrane-bound LDLR as compared to non-treated cells. Importantly, we achieved a 3-fold increase in LDL-C uptake, outperforming currently prescribed therapies. Analysis of off-target editing and inversion events demonstrated the genomic safety of this composition. We proceeded to testing our composition in lymphoblastoid cell lines derived from FH patients across a range of mutations. Using Western Blot, we found an increase in the mature glycosylated form of LDLR in all treated samples. Proof of concept experiments are currently underway in mouse models with elevated LDL-C. These findings support our CRISPR-based gene editing strategy of truncating regions likely responsible for rapid LDLR mRNA turnover to enhance its expression and boost LDL-C uptake. This unique approach could prove useful for a variety of hypercholesterolemia-related disorders.

1549 Novel AAV Capsid Identification and Characterization for Neuromuscular and Cardiac Indications

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Adeno-associated virus (AAV) mediated gene therapy continues to be a promising therapeutic path for various diseases, including monogenic neuromuscular and cardiac indications such as Duchenne muscular dystrophy (DMD) and Friedreich's ataxia (FA). However, the high systemic doses currently required to achieve widespread therapeutic benefit can pose potential safety risks. These risks could be decreased or eliminated if therapeutic benefit could be achieved using lower doses through a more targeted and efficacious vector. Numerous strategies exist to engineer novel AAV capsids for these types of improvements. We are continuing to employ rational design approaches to identify capsids exhibiting increased transduction of skeletal and cardiac muscles, while also de-targeting peripheral tissues, such as liver. Selection strategies include several *in vitro* and *in vivo* assays to increase potential translation to humans in future clinical trials. As individual candidates are identified, additional characterization around parameters such as neutralizing antibody escape, mechanism of action, and manufacturing will be evaluated more fully and benchmarked against other naturally occurring and engineered capsids. Previously, we have described AAV-SLB101, a novel AAV capsid that Solid is using in its SGT-003 pipeline program for DMD after observing a 2-4x increase in muscle transduction and ~0.5x decrease in liver tropism in comparison to AAV9 in mice (*DMD^{mdx}* and wild type) and non-human primates. This is one example of advances in AAV capsid engineering

and the possibility to change the course of AAV-based gene therapy for numerous neuromuscular and cardiac diseases, maintaining high transduction of target tissues at potentially lower doses while decreasing likelihood of safety events.

1550 A Phase 1, First in Human (FIH) Study of Autologous Anti-HER2 Chimeric Antigen Receptor Macrophage (CAR-M) in Participants (pt) with HER2 Overexpressing Solid Tumors

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Background: Macrophages are abundant in the solid tumor microenvironment (sTME) and can exhibit both pro- and anti-tumor functions. Macrophages can be redirected by CAR expression to phagocytose cancer cells in an antigen-specific manner. CAR-M can reprogram the sTME and present neoantigens to T cells, leading to epitope spreading and anti-tumor immunity. CT-0508 is comprised of autologous monocyte-derived proinflammatory macrophages expressing an anti-HER2 CAR. Pre-clinical studies showed that CT-0508 induced targeted cancer cell phagocytosis while sparing normal cells, decreased tumor burden, prolonged survival, and was safe and effective. Notably, anti-HER2 CAR-M treatment led to activation of the sTME, with infiltration of CD8+ and CD4+ T cells, NK cells, dendritic cells, and increased activated CD8+ tumor infiltrating lymphocytes. In a pre-clinical model of advanced solid tumor resistant to PD1 blockade, mice treated with anti-HER2 CAR-M combined with a PD1 blocking antibody demonstrated improved tumor control, overall survival, and TME activation compared to either treatment alone, indicating synergy and capacity for CAR-M to sensitize solid tumors to checkpoint blockade. **Methods:** This Phase 1, FIH study is evaluating safety, tolerability, cell manufacturing feasibility, trafficking, TME activation, and preliminary evidence of efficacy of investigational product CT-0508 in 18 pt with locally advanced (unresectable) /metastatic solid tumors overexpressing HER2. Pt previously treated with anti-HER2 therapies are eligible. Filgrastim mobilized autologous CD14+ monocytes are collected by apheresis, followed by manufacturing and cryopreservation. Group 1 pt (n = 9; enrollment complete) received fractionated doses over Days 1, 3, and 5. Group 2 pt (n = 9) receive CT-0508 as a single infusion on D1. Additional cohorts include: CT-0508 co-administered with pembrolizumab and CT-0508 monotherapy administered intraperitoneally in pt with peritoneal predominant disease. Correlative assessments include pre- and post-treatment biopsies and blood samples for safety (immunogenicity), trafficking (qPCR, RNA in situ hybridization), CT-0508 persistence in blood and tumor, target antigen engagement, TME modulation (single cell RNA sequencing), immune response (TCR sequencing) and others.

1551 Immunoadsorption Plasmapheresis for the Removal of Plasma Immunoglobulins to Enable Repeat Dose Administration with an AAV5 Gene Therapy Vector

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The investigation of adeno associated virus (AAV) vectored gene therapies has increased exponentially over the past decade for the treatment of monogenic disorders. However, the presence of pre-existing anti-AAV neutralizing antibodies (AAV NAb) may limit the efficacy of gene therapy. Moreover, a first administration of an AAV vector induces high titers of treatment emergent AAV NAb, which may compromise repeat dose administration with the same vector. Depletion of AAV NAb by immunoadsorption plasmapheresis (IAP) is a strategy that could allow successful vector administration in recipients with either pre-existing or treatment-emergent antibodies. The objective of this study was to evaluate the effectiveness of IAP to remove AAV serotype 5 (AAV5) NAb from animals sensitized by an initial gene therapy dose. Five cynomolgus macaques (*Macaca fascicularis*) were included in this study; four were sensitized by administration of an AAV5 capsid encoding for the beta subunit of cyno chorionic gonadotropin (AAV5-βCG) at a dose of 6E13vg/kg, and one control animal was naïve. All were subjected to IAP for a minimum of 1 day of 4 runs (plasma volume exchanges) to a maximum of 3 days of 3 runs. All 5 animals were challenged with the exact same AAV5 capsid encoding a different protein, human factor IX (AAV5-hFIX) at a dose of 6E13 vg/kg, administered within 10 minutes of the last run IAP. Efficacy of the IAP procedure was functionally evaluated by laboratory measures of plasma IgG and AAV5 total binding antibody (AAV5 TAb) titer, hFIX plasma protein concentration, and quantitation of vector genomes and transcripts in liver tissue. Maximal depletion of AAV5 TAb titer (>99%) was achieved in two animals resulting in a nadir titer of 61 and 59. These two animals achieved approximately 25% and 50% of hFIX plasma protein levels, respectively, compared to the naïve animal (0.8 IU/mL), and a proportional percentage of vector genome copies measured in liver tissue compared to the naïve animal (1.6 x 10^7 cp/µg tissue). Following a variable number of IAP sessions (plasma volume exchanges over consecutive days) and administration of the AAV5-hFIX challenge dose, there was significant perturbation of hematological and biochemical blood parameters; however, all parameters returned to baseline levels within hours or days of the procedure. These results demonstrate the viability of IAP as an immune modulation procedure to deplete AAV5 capsid-specific

antibody titers sufficient to allow repeat dose administration. As such, IAP may enable AAV-based vector gene therapy in patients currently excluded from gene therapy clinical trials or commercial product use due to pre-existing antibodies. Furthermore, additional evaluation of the efficacy of this procedure may be worthwhile in subjects with pre-existing antibody titers resulting from natural exposure to AAV infections, which result in lower antibody titers than the treatment-emergent titers observed here.

1552 Extracellular Vesicles as Efficient Delivery Modality for shRNA

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Introduction: Owing to their ability to target virtually any RNA within a cell, RNA interference (RNAi)-based drugs offer an immense therapeutic potential. However, the hydrophilic nature and low bioavailability of naked RNAi agents require a delivery strategy. To date, a plethora of different strategies exist, including encapsulation into synthetic lipid nanoparticles. However, their application suffers from toxic side effects of excipients and predominant hepatic accumulation. Here, we propose the use of extracellular vesicles (EVs), which are naturally occurring nanoparticles that benefit from immune tolerance and the ability to cross biological barriers, as RNAi delivery vehicles. By applying state-of-the-art engineering approaches, we produced EVs that harbor therapeutic amounts of short-hairpin RNA (shRNA) for potent delivery to recipient cells. Methods: EV-producing cells were genetically modified to express 1) a shRNA of interest, 2) AGO2 as an RNA binding protein fused to an EV sorting domain (CD63), and 3) a fusogenic viral glycoprotein to ensure potent delivery to the cytosol of recipient cells. EVs were isolated from the conditioned media of producer cells and purified by size exclusion chromatography. Subsequently, their contents and properties were analyzed by quantifying the shRNA copies present in EVs (RT-qPCR) and by assessing their downstream functionality in terms of eliciting silencing on the mRNA (RT-qPCR) and protein (Western blot) level in recipient cells *in vitro*. Results: Here, we show that engineered EVs are potent delivery modalities for shRNA *in vitro*. Applying our approach to load shRNA targeting Gapdh (shGapdh), we detected up to 1 shRNA molecule per 3 EVs. Strikingly, our loading strategy boosted the number of shGapdh molecules per EV by 160-fold in comparison with stochastic loading by overexpression. In an *in vitro* setting, these EVs achieved an IC50 of 0.17 nM and Gapdh silencing efficiencies up to 90% thereby competing with state-of-the-art transfection of plasmid DNA encoding for shGapdh (Lipofectamine 2000). Apart from shGapdh, we obtained similar results for a shRNA targeting GFP thus demonstrating the versatility of our approach. Furthermore, moving towards a therapeutically relevant target, we are currently evaluating engineered EVs for shRNA delivery *in vivo*. Conclusion: Engineered EVs present a viable option for the delivery of RNAi agents, such as shRNA. Importantly, we are confident that the strategy developed here can be repurposed for the delivery of other small RNA therapeutics including siRNAs. Additionally, considering the modifiable nature of EVs, future studies will achieve cell or tissue specific targeting of EV-encapsulated RNAi drugs therefore opening up therapeutic applications beyond the liver.

1553 Use of *In Vivo* Electroporation for Gene Delivery to the Airways

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Cystic Fibrosis (CF) is a multisystem monogenetic disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR). While great progress in treatment has been made in recent years due to the development of novel therapeutics that can post-translationally increase CFTR activity in most patients, almost 10% of patients do not benefit from these drugs due to their specific CFTR mutations (namely Class I nonsense and many Class V splicing mutants). As such, methods to increase CFTR expression and activity are still needed to provide relief for these patients. Gene therapy is seen as a likely answer to this need. A number of different approaches for CF gene therapy have been developed and tested over the years, but none have yet elicited any significant benefit in patients. The search for effective delivery methods continues. Electroporation is a gene delivery technique that uses electrical fields to create transient pores in the cell membrane that allow the entry of normally impermeable macromolecules into the cytoplasm. We have shown that the electric pulses needed for electroporation can be applied across the chest of an anesthetized animal (mouse, rat, rabbit, and pig) following intratracheal delivery of plasmid DNA and lead to effective and safe gene transfer. However, most of our previous studies have focused on delivery to the alveolar epithelium for short-term gene expression to treat acute injuries; we have not evaluated gene transfer to the airways to any significant degree. To observe gene transfer to the airways, we used plasmids that express GFP or GFP-CFTR fusion proteins for short or long periods, based on the promoters driving expression. Robust gene delivery and expression was seen throughout both large and small airways in the mouse following electroporation. While the CMV promoter expressed for up to 7 days, by 14 and 28 days, no expression was detected. By contrast, the human UbC promoter gave robust gene expression at all times. Approximately 80% of epithelial cells in the airways (n>300 airways, >30 mice) showed GFP expression for either promoter and gene product at early times and UbC constructs continued to show the same expression levels at 28 days post-transfer. Co-staining for cell-type specific markers indicated that approximately 1/3 of the productively transfected cells are ciliated and another 1/3 are secretory cells. Continuing analysis will better define the distribution of gene transfer and expression. We also tested whether electroporation could be used for efficient gene delivery to mucus-filled lungs, an unfortunate characteristic of the lungs of patients with CF. When plasmids expressing luciferase were electroporated into CCSP-betaENaC transgenic mice that show reduced airway surface liquid and abundant mucus, no difference in luciferase expression was detected compared to the gene transfer seen in their wild type littermates. Further, the distribution of gene transfer and expression in mutant and wild type mice was the same. Taken together, our results demonstrate

that electroporation can be used safely for gene transfer leading to short or long-term expression in the airway epithelium and suggest that this delivery approach could be viable for the treatment of CF.

1554 Investigating the Mechanism to Develop a Therapeutic Approach to Resetting Lung Molecular Clock During HIV-Mediated COPD

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People living with HIV (PLWH) are at an increased risk for chronic obstructive pulmonary disease (COPD), asthma, and poor health outcomes. COPD exacerbations increase with disease progression and predominantly occur at night or in the early morning hours, possibly due to circadian dysregulation in mucus physiology and lung inflammation. Circadian rhythms are near 24-hour biological oscillations mediated by changes in the expression of clock genes. Peripheral circadian clocks can rapidly become desynchronized with adverse environmental stimuli. We are investigating lung inflammation as a consequence of HIV-mediated disruption of the lung clock to identify the mechanism involved. Circadian dysregulation will be determined in a lung-specific HIV-tat transgenic mouse model. Our data suggest that HIV dysregulates the microRNAome to suppress circadian genes. The consequence of bronchial epithelial microRNAome dysregulation and its impact on the lung molecular clock will allow us to identify the miRNAs involved as well as the pathophysiology underlying increased lung inflammation in HIV with a long-term goal towards designing therapeutics to reset the circadian clock and decrease lung inflammation in PLWH.

1555 Engineering the Novel Type V-F CRISPR-Cas System as Versatile and Efficient Genome Editing Tools Delivered by a Single AAV

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Background: The type V-F CRISPR-Cas12f system is a strong candidate for therapeutic applications due to the compact size of the Cas12f proteins. Recent studies have shown the huge potential of Cas12f for genome editing in eucaryotic cells. Nevertheless, more efficient CRISPR-Cas12f systems with minimal protospacer adjacent motif (PAM) constrains remain to be discovered. Here we developed novel Cas12f1 as a gene-editing tool delivered by a single AAV. **Methods**: We employed a computational pipeline to annotate Cas12f1 orthologs, CRISPR array, tracrRNAs, and PAM preferences by data mining assembled bacterial genomes. An EGFP-activation reporter system was designed and used to screen functional CRISPR-Cas12f1 systems in human cells. Protein engineering combined with sgRNA optimization were used to generate enhanced Cas12f1 variants with high editing efficiency, high fidelity and broad PAMs. By leveraging small molecule-stabilized domain, a tunable Cas12f1 system was developed. Furthermore, catalytically inactive Cas12f1 variants were constructed and applied in epigenetic editing and gene regulation. **Results**: We identified six undocumented Cas12f1 proteins with nuclease activity in mammalian cells from assembled bacterial genomes. Among them, HE1Cas12f1 and HE6Cas12f1 (~420 aa only, Fig. 1a), which respectively target 5' T-rich and 5' C-rich PAMs, showed the highest editing activity. Through protein and sgRNA engineering, we generated enhanced HE1Cas12f1 (enHE1Cas12f1) and enHE6Cas12f1 variants, with 5'-TTN and 5'-CCD (D = not C) PAMs respectively, exhibiting much higher editing efficiency, broader PAMs, and comparably high fidelity, compared with the engineered variant of Un1Cas12f1 (Un1Cas12f1_ge4.1) (Fig. 1b-1d). Furthermore, we generated a small molecule-inducible enHE1Cas12f1 and demonstrated its activity with paired sgRNAs *in vivo* by single adeno-associated virus (AAV) delivery, wherein a large fragment containing the exon51 of *DMD* (~1,700 bp) was deleted and expression of dystrophin protein was sufficiently rescued in DMD mice generated by deleting mouse exon52 and replacing mouse exon51 with human exon51 (Fig. 2a-2b). Finally, dead enHE1Cas12f1-based epigenetic editing and gene activation could also be efficiently achieved in mammalian cells (Fig. 2c). **Conclusions:** Our data suggested that enHE1Cas12f1 and enHE6Cas12f1 are established as compact and versatile gene editing tools for basic research with remarkable promise for therapeutic applications.

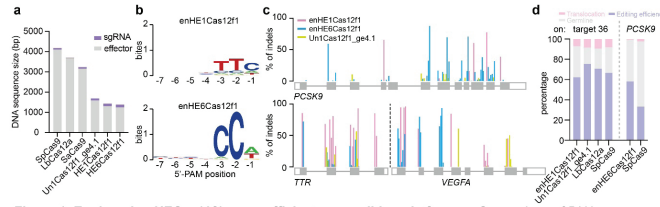

Figure 1. Engineering HECas12f1 as an efficient gene editing platform. a, Comparison of DNA sequence sizes of different CRISPR systems. **b,** WebLogos of the PAM sequences. **c,** Comprehensive validation of genomic editing efficiency of Cas12f1s in human cells. **d,** PEM-seq genome-widely quantified the translocation efficiencies induced by off-target indels of enHE1Cas12f1 and enHE6Cas12f1.

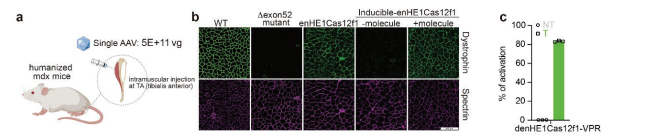

Figure 2. Applying enHE1Cas12f1 for *in vivo* gene editing and *in vitro* gene activation. a, Overview of intramuscular injection of single AAV system in humanized mdx mouse. **b,** DMD immunofluorescence staining. **c,** denHE1Cas12f1-VRP mediated gene activation in HEK293T cells.

1556 Gene Therapy Development Using an Inducible Knockout Mouse Model of NGLY1 Deficiency

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NGLY1 deficiency is an ultrarare autosomal recessive deglycosylation disorder caused by biallelic mutations in the *NGLY1* gene encoding cytoplasmic peptide:N-glycanase (NGLY1). NGLY1 is ubiquitously expressed and normally functions as a de-N-glycosylating enzyme that, together with the 26S proteasome, degrades misfolded N-glycosylated proteins in the cytoplasm. Patients suffer from multisystemic involvement including developmental delay, motor function impairment, hypotonia and liver dysfunction, and the neurological

manifestations represent the major disease burden. Currently, there is no treatment for this disease. Unlike in humans, NGLY1 deficiency in mice is embryonically lethal, posing a significant challenge to studying the disease mechanisms and testing AAV-based gene therapy *in vivo*. In this study, we obtained an *Ngly1* inducible knock-out (iKO) mouse model and demonstrated the therapeutic efficacy of brain-directed *NGLY1* gene replacement therapy. The iKO mice carry a loxP-flanked *Ngly1* exon and a tamoxifen-inducible, ubiquitously expressed *cre* transgene that enables tamoxifen-dependent *Ngly1* knockout. We first optimized a neonatal tamoxifen treatment regimen to induce *Ngly1* knockout in multiple tissues. Consequently, the tamoxifen-treated iKO mice exhibited impaired motor function, progressive kyphosis, lower body weight, and a reduced life span. We hypothesized that broadly restoring NGLY1 in the brain via systemic AAV-mediated gene delivery can alleviate the disease phenotype. At 7 weeks old, iKO mice were treated with rAAV.PHPeB expressing a codon-optimized human *NGLY1* cDNA via tail vein injection. We found that the rAAV treatment significantly improved motor function as measured by rotarod test and CatWalk gait analysis. Kyphosis was largely corrected as revealed by X-ray radiography. Body weight and survival were also improved. Our data suggests that brain-directed *NGLY1* gene replacement via systemic delivery is a promising therapeutic strategy for NGLY1 deficiency. Although the superior mouse CNS tropism of AAV.PHPeB vector used in this study does not translate to primate, emerging AAV capsids with enhanced human CNS tropism will enable future translational studies. In addition, our study exemplified using an iKO mouse model to overcome embryonic lethality and to test *in vivo* gene therapy. (#Co-corresponding authors)

1557 Intravitreal Delivery of Ixo-vec for Neovascular Age-Related Macular Degeneration Results in Widespread Expression of Aflibercept in Cynomolgus Monkey Eyes as Confirmed Using *In-Situ* Hybridization

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Neovascular age-related macular degeneration (nAMD) is an advanced form of age-related macular degeneration (AMD) that is responsible for approximately 90% of cases of severe vision loss due to AMD (Shao, 2016). Vascular endothelial growth factor (VEGF) is a key component in the development and progression of nAMD, and current treatment strategies require frequent anti-VEGF bolus injections. Ixo-vec does not replace or modify an abnormal gene, but instead turns cells into a biofactory that continuously delivers anti-VEGF to the retina with a single IVT injection. Utilizing an AAV2.7m8-based vector optimized for broad transduction of the retina and ubiquitous transgene expression, Ixo-vec provides sustained aflibercept protein levels in non-human primate (NHP) ocular fluids out to 2.5 years after injection (Kiss, 2021) and in humans as demonstrated in the OPTIC trial. To improve our understanding of AAV vector genome (vg) distribution and mRNA expression, the per cell intraocular distribution of IVT-delivered Ixo-vec at 3E10 and 1E11 vg/eye (human equivalent dose (HED) 6E10 and 2E11 vg/eye) was evaluated in a GLP compliant 3 month-long study in Cynomolgus monkeys. The doses evaluated were well tolerated, with observations limited to dose-related non-adverse low-grade inflammation, and non-adverse infiltrates. The intraocular pressure in treated eyes was normal (≥14mm Hg) throughout the study. The no-observed-adverse-effect-level was established at 1E11 vg/eye dose (HED 2E11 vg/eye). The pattern of ocular expression was evaluated using BaseScope, an in-situ hybridization assay optimized to detect AAV vector genomes (vgs) and aflibercept mRNA in the eyes of NHPs. The assay utilized a probe designed against the engineered promoter for the detection of vgs, and a 1-ZZ paired probe flanking the splicing junction within the cassette promoter elements for the detection of aflibercept mRNA. The mRNA aflibercept-specific probe only generates a signal after the 5' UTR intron has been spliced, which allows for the precise detection of transgene mRNA without vector DNA interference. Evaluation of vg distribution identified widespread vg presence in anterior and posterior ocular tissues. In the retina, the presence of vector DNA was restricted to the macula and periphery, regions where the inner limiting membrane (ILM) are the thinnest. In these regions, cells of the retinal ganglion cell (RGC) layer, inner nuclear cell layer (INL), and photoreceptor (PR) layer contained vector DNA. In the mid-retina, where the ILM is the thickest, vgs were bound to the ILM and not found within the cells of the deeper layers of the retina. The presence of aflibercept mRNA correlated well with vg DNA that was present intracellularly. Aflibercept mRNA was identified in anterior and posterior tissues mirroring the intracellular presence of vgs. In the retina, the presence of transgene mRNA was restricted by the ILM to cells within the RGC, INL, and PR layers of the macula and periphery. Transgene mRNA was not detected in the mid-retina where the ILM is the thickest. The study demonstrated IVT administration of Ixo-vec at doses 3E10 and 1E11 vg/eye (HED 6E10 and 2E11 vg/eye) resulted in widespread distribution of aflibercept mRNA with the primary localization in the macula and peripheral retina. The results demonstrate widespread distribution of aflibercept mRNA expression across ocular cell types and regions of the eye does not result in adverse ocular changes and could contribute to the maintenance of durable therapeutic ocular aflibercept levels.

1558 Microfluidic Droplet Cell Mechanoporation Enables Highly Efficient Genome Editing in Human Primary Immune Cells

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Cell-based therapy has shown compelling clinical evidence, and it has changed the paradigm of cancer treatment. A representative example of cancer immunotherapy includes chimeric antigen receptor-engineered T cell (CAR-T) therapy. In order for T cells to be given therapeutic activity, external genetic molecules such as mRNA, plasmid DNA, and CRISPR-Cas RNP must be internalized into T cells. For cell gene modification, benchtop methods such as viral transduction and electroporation have been widely used; however, these methods are

limited by high cost, low throughput, safety concerns, and/or loss of cell functions. To overcome these drawbacks, we present a microfluidic intracellular delivery platform for non-viral genome editing with microdroplet-enabled cell mechanoporation.

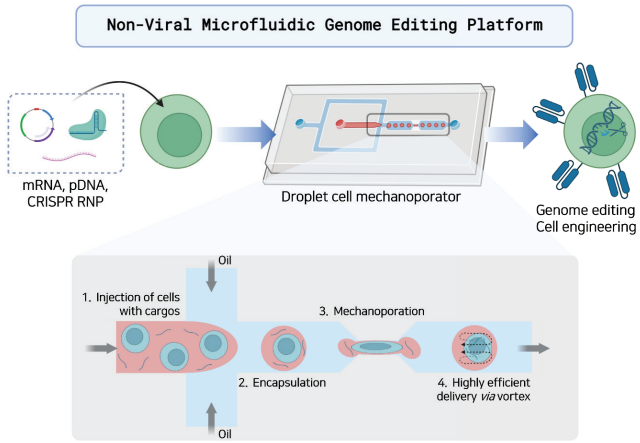

Figure 1. Schematic illustration of genome editing process *via* droplet cell mechanoporator.

In this approach, cells and target genetic materials are co-encapsulated into droplets, and when they go through a small constriction, mechanical cell stretching temporarily permeabilizes the cell membrane. The external cargos are internalized by recirculation flows developed in the droplet through the created membrane discontinuities, allowing for highly efficient delivery while maintaining high cell viability. With the use of our platform, we were able to internalize GFP-expressed mRNA and plasmid DNA with extremely effective cell transfection efficiency. By delivering CRISPR-Cas9 RNPs for non-homologous end joining (NHEJ), double NHEJ, and homology directed repair (HDR), we also demonstrated much greater genome editing efficiency compared with electroporation and lipofection. In summary, high delivery efficiency, high scalability, low analyte consumption, and low-cost operation for CRISPR-based genome editing were demonstrated, validating the high potential of the proposed platform for applications in cell-based therapies.

1559 Digital Droplet PCR Assay Robustness

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Vector genome (VG) titer is arguably the most important critical quality attribute of a therapeutic AAV. Ideally, an accurate and precise VG titer method is used throughout the manufacturing process to ensure product quality, stability and, most importantly, clinical dosing decisions. Therefore, establishing a robust vector genome titer method early in AAV product development is absolutely essential. Through exhaustive development and optimization of a droplet digital PCR (ddPCR)-based method for vector genome titer, we consistently achieve inter-assay variability of < 15% for Phase 1 studies across multiple programs. This was achieved through the addition of automation, optimized primer/probe design and method workflow harmonization. A failure mode effect analysis was performed on the optimized method to identify critical method parameters and was followed by robustness testing to confirm accuracy and precision during these critical steps of the method. The end result is a robust VG titer method suitable for the entire product development lifecycle from pre-clinical through registrational studies.

1560 Development of a Gene Therapy for UBA5 Deficiency

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Ubiquitin-like modifier activating enzyme 5 (UBA5) deficiency is an ultra-rare autosomal recessive neurological disorder. UBA5 is the E1 activating enzyme in a ubiquitin-like post-translational modification system called ubiquitin-fold modifier 1 (UFM1) cascade. Compound heterozygous mutations in UBA5 lead to disruption of UFM1 cascade and cause early infantile-onset encephalopathy; however, the role of UBA5 and UFM1 cascade in central nervous system is not understood. UBA5 patients present with infantile spasms, failure to thrive, hypotonia, developmental delay, and microcephaly. Patients develop movement disorders, have intellectual deficit, vision defects, epilepsy and some do not acquire language skills. Life span is variable. Currently there is no treatment for UBA5 deficiency. We designed self-complementary AAV9 vectors encoding UBA5 under four different promoters to determine the most effective transgene cassette to express UBA5 ubiquitously and restore function of UFM1 system. All vectors encode human UBA5 with a C-terminal HA tag. AAV vector functionality was confirmed by transient transfection of UBA5-knockout-HEK293T cells, which were generated by CRISPR/Cas9 genome editing. Our in-vitro results indicate all four vector constructs restore expression of UBA5 at higher-than-normal levels and importantly restore UFM1 system functionality.Considering that there is no viable mouse model of UBA5, we performed a biodistribution/safety study in wild-type mice by neonatal intracerebroventricular infusion of four different AAV9-UBA5 vectors. Mice were sacrificed at 6 weeks of age and western blot analysis confirmed UBA5 expression in the brain in all AAV-treatment groups. Importantly, AAV expressed UBA5-HA *in vivo* was shown to interact with its molecular partners to activate the UFM1 pathway. Next steps are to evaluate the functionality of the four AAV9-UBA5 vectors in UBA5 patient fibroblasts, followed by an FDA meeting around toxicology study design to establish safety prior to first-in-human clinical trials.

1561 Cell-Type Specific Reduction of Prion Expression in Neurons and Astrocytes Using Engineered Zinc Finger Transcriptional Regulators

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Prion disease is a fatal neurodegenerative disorder caused by misfolding and aggregation of the prion protein, PrP, encoded by the *PRNP* gene. Cellular PrP is ubiquitously expressed throughout the body and *PRNP* transcripts are abundant in both neurons and glia. Nevertheless, several lines of evidence from prion-infected mouse models suggest that neuronal PrP expression is necessary and sufficient for neurotoxicity and disease progression. We are developing a potential single administration therapeutic approach using zinc finger repressors (ZF-Rs) to lower the expression of brain PrP. To evaluate the contribution of different cell types to prion expression at the bulk and single-cell level in the brain, we generated AAV vectors expressing a highly potent and specific prion-targeted ZF-R under the control of one of three different promoters for neuron (hSYN1), astrocyte (GfaABC1D), or non-cell type specific (CMV) expression. In cultured mouse primary neurons and astrocytes, ZF-R treatment reduced *Prnp* mRNA in a dose-dependent manner. While hSYN1-driven expression was restricted to neurons, CMV- and GfaABC1D-driven expression were found in both cell types. In wildtype mice treated with these constructs, all tested promoters resulted in at least 50% bulk prion mRNA reduction (hSYN1 ≥ CMV > GfaABC1D) when compared to the control group. Brain hemispheres from the same experiment were analyzed by multiplexed RNAscope and immunohistochemistry, and cortex was analyzed by single-nucleus transcriptomics. For all promoters, a strong negative correlation between ZF-R and *Prnp* expression was observed at the single-cell level throughout the brain. Neuron-specific expression was observed for the hSYN1 promoter in all brain regions examined. For the CMV group, heterogenous expression was observed, primarily in neurons and astrocytes. In contrast to previous reports, the GfaABC1D promoter did not appear to drive expression in astrocytes, but rather in neurons, albeit more weakly than hSYN1. Hence, we initiated an effort to identify enhancers capable of brain-restricted expression in neurons and astrocytes. First, from existing mouse and human single-cell transcriptomic profiles, we identified genes that are expressed in both neurons and astrocytes but lack expression in liver. We then extracted putative upstream enhancer regions from the human orthologs into multiple distinct segments suitable for AAV packaging. AAV-promoter-ZF-Rs were screened in primary mouse cortical neurons, primary rat astrocytes, and human iPSC-derived astrocytes. Transduction efficiencies and promoter specificity were evaluated using confocal imaging for GFP reporter expression and RT-qPCR for ZF-R and *Prnp* expression. Top enhancer segments exhibited expression and potent *Prnp* reduction in both neurons and astrocytes. Based on these encouraging results, top candidates will be evaluated *in vivo* to assess cell-type and tissue-specific ZF-R expression, *Prnp* reduction, and CSF PrP knockdown.

1562 Treatment of Huntington's Disease by Inhibition of Caspase-6 Cleavage Using CRISPR Base Editing

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Huntington's disease (HD) is a neurodegenerative condition with autosomal dominant inheritance, characterized by a progressive decline in cognitive, motor, and behavioral features. HD is caused by the expansion of a polyglutamine-encoding 'CAG' repeat within exon 1 of the Huntington (HTT) gene which results in the formation of mutant protein aggregates that are mainly toxic to the cells in the striatum and cortex regions of the brain, consequently leading to neuronal loss and dysfunction. Current treatments for HD are purely symptomatic and hence there is a need for therapies targeting the underlying genetic defect. Although CRISPR Cas systems have been utilized to induce targeted modifications in genomic DNA, their reliance on creating double stranded breaks (DSBs) in DNA to achieve editing is a limitation to their use from a therapeutic standpoint. Moreover, directly correcting the disease-causing mutation is complicated due the repetitive nature of the mutation causing HD and a non-allele specific reduction of HTT expression is undesirable because the functions of the wild-type HTT protein are not well understood. Studies have shown that cleavage of the mutant HTT (mHTT) protein at the predicted caspase-6 binding site encoded by HTT exons 12-13, plays a key role in HD pathogenesis. Caspase-6 cleavage of mHTT generates highly toxic N-terminal and C-terminal derivatives that are responsible for the development of symptoms characteristic of HD. In this work, we utilized CRISPR-SKIP, a base editing strategy to induce permanent exon skipping by introducing C>T or A>G mutations at the conserved splice acceptor site in genomic DNA, to exclude the Caspase 6 binding domain from mature mRNA HTT transcripts. First, we characterized the editing outcomes of base editors that disrupt the consensus splice acceptor of HTT exon 13 in cultured human cells and analyzed the patterns of mRNA expression leading to the removal of Caspase 6 cleavage site. Next, we utilized split-intein base editing technology to deliver the base editing systems into the striatum of YAC128 HD mouse model using AAV9. Our approach successfully mutated the splice acceptor of HTT exon 13 in vivo and facilitated the survival of these edited neurons. Moreover, we also report a preservation of brain volume and decreased levels of toxic mHTT inclusions in the striatum at 18 months of age in the BE- treated transgenic mice- a promising in vivo therapeutic outcome that supports further development of this technology for the treatment of HD. Overall, our work supports the potential of AAV-encoded split BEs for the treatment of Huntington's disease.

1563 mRNA Cell Engineering Enables Rapid Prototyping of Macrophage Gene-Editing Strategies for Cancer Immunotherapy Applications

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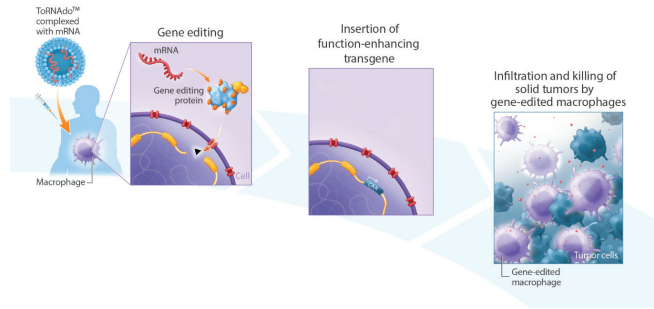
Macrophages' ability to infiltrate solid tumors and engage in both direct killing of cancer cells and recruitment of other immune cells has made them a promising target for development of next-generation cancer immunotherapies. The innate ability of macrophages to ingest foreign genetic material also facilitates their engineering with formulated nucleic acids, including mRNA. The oncoantigen tyrosine-protein kinase transmembrane receptor ROR1 has garnered interest for its minimal expression in healthy adult cells and overexpression in many malignancies, including solid tumors associated with ovarian, lung, and triple-negative breast cancer. Here, we present an mRNA-based platform for rapid prototyping of macrophage engineering approaches. We show mRNA delivery to peripheral blood mononuclear cell (PBMC) and iPS cell-derived macrophages for gene editing prototyping and functional assessment of encoded proteins. To develop this platform, we transfected macrophages with unmodified or 5'-methoxyuridine (5-moU)-containing mRNA encoding green fluorescent protein (GFP). Both mRNAs resulted in more than 95% of cells displaying GFP within 4 hours. We next designed an ROR1-targeting CAR with a CD3 zeta activation domain and 4-1BB costimulatory domain. Transfection of mRNA encoding the ROR1-CAR yielded 70% CAR-expressing cells, as measured using PE-labelled ROR1. We then compared ROR1 affinity of rabbit and humanized binding domains and found that the humanized binding domain displayed a 2.5-fold increase in affinity as measured by flow cytometry using PE-labelled ROR1. The human receptor domain, but not the rabbit domain, demonstrated activation when bound to ROR1 as assessed by immunofluorescence of CD3 zeta phosphorylation. We also assessed the mRNA-encoded ROR1-CAR's functionality by measuring killing of ROR1-expressing SKOV-3 ovarian cancer cells. Both the rabbit and humanized ROR1 domains of the CAR displayed significantly increased cytotoxicity towards SKOV-3 cells when compared with untransfected macrophages after a 24-hour co-culture at a 5:1 effector-to-target ratio ($p<0.01$). We then inserted the ROR1-CAR sequence into the AAVS1 safe harbor locus of iPS cells under the control of an SFC promotor, isolated 5 biallelic-inserted lines, and differentiated them into macrophages. These results demonstrate an mRNA-based platform for rapid prototyping of macrophage engineering approaches. Transfection of macrophages with mRNA encoding a chimeric antigen receptor (CAR) targeting ROR1 resulted in functional expression *in vitro*, facilitating optimization of the antibody and co-stimulatory domains to improve protein binding affinity and immune activation. This platform thus enables the assessment and validation of novel macrophage gene editing strategies and is being explored for the development of macrophage-engineering therapies for solid tumor applications.



1564 *Ex Vivo* Prime Editing Corrects the Sickle Cell Allele in Hematopoietic Stem Cells and Corrects Disease Phenotypes in Mice

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Sickle cell disease (SCD), a deadly monogenic disorder affecting millions of individuals, is caused by an A•T-to-T•A transversion mutation in the β-globin gene (*HBB*). We optimized a prime editing system to directly correct the SCD allele (HBB^S) to the wild-type allele (HBB^A) with high ratios of the desired edit to indel byproducts. Sickle cell patient hematopoietic stem and progenitor cells (HSPCs) electroporated with PEmax mRNA, an engineered pegRNA (epegRNA), and a nicking single-guide RNA (sgRNA) yielded up to 41% conversion of HBB^S to HBB^A. Seventeen weeks after transplantation of prime edited HSPCs into immunodeficient mice, high-level correction of HBB^S was maintained in all donor-derived cells with no alterations detected in the frequency of engraftment, hematopoietic differentiation, or lineage maturation compared to unedited healthy donor HSPCs. On average, 42% of human erythroblasts and reticulocytes isolated 17 weeks after transplantation of prime-edited HSPCs from four SCD patient donors contained monoallelic or biallelic *HBB* correction, exceeding the levels predicted to be required for therapeutic benefit in patients. Red blood cells derived from prime-edited SCD HSPCs contained significantly reduced levels of sickle hemoglobin (HbS), had a proportionate increase in normal adult hemoglobin (HbA) of up to 42%, and were resistant to hypoxia-induced sickling. An extensive analysis of over 100 candidate off-target sites nominated by an unbiased genome-wide experimental approach detected minimal off-target editing from all prime editing components used for the corrective edit. Our findings represent one of the first PE-based therapeutic strategies for HSCs, suggesting the potential of a one-time treatment for sickle cell disease that directly corrects pathogenic HBB^S to wild-type HBB^A, does not require delivery of any viral or non-viral DNA template, and minimizes undesired consequences associated with DNA double-strand breaks.

1565 Development of a Self-Complementary AAV.U7snRNA Vector for Efficient Dystrophin Exon 53 Skipping

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Duchenne Muscular Dystrophy (DMD) is a genetic disease caused by mutations in the *DMD* gene, encoding dystrophin. Of these mutations, roughly 80% are amenable to exon skipping, a therapeutic approach in which specific exon(s) are excluded from the mature *DMD* mRNA to restore the open reading frame disrupted by mutations. The result is a nearly full length and functional dystrophin protein. Here, we describe development of AAV.3XU7.Ex53, a self-complementary (sc) AAV vector expressing three antisense sequences on a modified U7 small-nuclear RNA (snRNA) scaffold that achieves efficient skipping of dystrophin exon 53 *in vitro* and *in vivo*. Multiple anti-sense sequences were designed to target exonic splicing enhancer sequences in intron 52 and exon 53, and the splice acceptor site in intron 52. Chosen antisense sequences were linked to a modified U7 snRNA and cloned into an AAV.U7 cassette plasmid. Sequences were tested for skipping efficiency with plasmid transfection, and the top sequences were packaged into AAV for further evaluation. AAV vectors expressing a single U7 snRNA induced modest levels of exon 53 skipping in cultured human rhabdomyosarcoma (RD) cells, as assayed by digital droplet PCR. However, the combination of two separate U7 snRNA cassettes into one AAV vector synergistically increased exon skipping efficiency, which was further amplified with the addition of a third unique U7 snRNA cassette. The final construct, AAV.3XU7.Ex53, demonstrated efficient exon 53 skipping of up to 90% in RD cells with comparable expression of all three U7 snRNAs. Analysis of vector genomes from AAV.3XU7.Ex53 vector preps via TapeStation and PacBio long read sequencing revealed high quality genome integrity, indicating the repeated U7 cassettes and hairpins within the snRNA did not negatively impact the packaging of full length scAAV genomes. Given our promising *in vitro* data, we performed an *in vivo* proof-of-concept study in hDMDdel52/*mdx* mice, which express human *DMD* without exon 52 and are amenable to exon 53-skipping therapies. Four weeks post AAV infusion, skipping efficiency of exon 53 in heart was 70% (low-dose) and 85% (high-dose), which corresponded to 31.0% and 59% of wildtype (C57Bl/6J) levels of dystrophin protein, respectively. In the gastrocnemius, 36% (low-dose) and 61% (high-dose) skipping resulted in 11% and 30.4% of wildtype protein. Analysis of skeletal muscle histopathology with Sirius red and embryonic myosin heavy chain immunofluorescence revealed that fibrosis and muscle regeneration were significantly reduced at either dose compared to mice treated with a scramble U7 snRNA vector. Together, these data demonstrate the potential of AAV.3XU7.Ex53 as a therapy for DMD patients amenable to exon 53 skipping.

1566 Development of CRISPR-Cas12f Gene Therapy for Androgenic Alopecia via Targeting of SRD5A2 Gene

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Androgenic alopecia is a common form of hair loss that can lead to baldness and requires medical attention. A major cause of androgenetic alopecia is a conversion of testosterone (TS) to dihydrotestosterone (DHT) by the enzymatic activity of steroid type II 5-alpha-reductase (SRD5A2). Excessive conversion of TS to DHT by SRD5A2 causes damage to hair dermal papilla cells (DPC) that leads to apoptosis of hair DPC and male-pattern hair loss. Accordingly, SRD5A2 has been a major target for androgenic alopecia therapy, and there have been attempts of therapeutic applications of CRISPR-Cas9 systems to target SRD5A2. While Cas9 is an effective genome editing system that is commonly utilized, its large gene size is one of the technical problems for in vivo delivery via virus. To address the delivery issue, we sought to develop SRD5A2 targeting genome editing by Cas12fl, of which the compact size enables efficient delivery by virus like AAV. Furthermore, we intended to deliver CRISPR-Cas system via AAV virus and lipid nanoparticle, that can deliver to mouse by intradermal injection. To this end, we conducted SRD5A2 gene knock-out in HEK293, human DPC, and NIH3T3 via delivering Cas12fl by plasmid vectors and lentiviral delivery system. We observed that delivery of Cas12fl to human cells and mouse cells induced significant indel frequencies comparable to that of Cas9. Our results suggested that CRISPR-Cas12f system can be utilized for genome editing of SRD5A2 as a mean to develop therapeutics against androgenic alopecia treatment.

1567 Developing and Characterizing an AAV Transgene Cassette That Expresses eCD4-Ig and TPST2

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The development of antiretroviral therapy (ART) has significantly prolonged the lifespan of people living with HIV-1. Despite the various ways ART works to prevent viremia, infected individuals still harbor latently infected CD4+ T cells that serve as viral reservoirs. Recent advancements in gene therapy have demonstrated promising results which may be used to treat HIV-1. One of many gene therapy delivery systems is the use of adeno-associated virus (AAV) vectors which have shown to efficiently deliver and express anti-HIV inhibitors and antibodies. Our previous work developed and characterized the anti-HIV inhibitor, eCD4-Ig which contains very broad and potent neutralizing features and the ability to elicit effector functions. eCD4-Ig is composed of the CD4 immunoglobulin domains 1 and 2, an antibody Fc domain, and a tyrosine-sulfated coreceptor-mimetic peptide which mimics the coreceptor of all HIV-1 and simian immunodeficiency

virus (SIV) isolates. Sulfation of the coreceptor-mimetic peptide is mediated by the enzyme TPST2. Due to its ability of binding to conserved regions of Env, eCD4-Ig efficiently neutralized 100% of a diverse panel of neutralization-resistant HIV-1, HIV-2, and SIV isolates. AAV-expressed eCD4-Ig provided durable protection to rhesus macaques after challenges with SHIV-AD8 or SIVmac239. Additionally, we were able to sustain expression of eCD4-Ig for over a year. Despite observing sustained eCD4-Ig expression in vivo, reaching therapeutic concentrations of eCD4-Ig remains a challenge. The initial work for expression of eCD4-Ig and TPST2 consisted of using two separate AAV vectors, each containing a single transgene cassette. Our new strategy uses a single AAV vector that expresses eCD4-Ig and TPST2 from a single transgene cassette. Initially, we engineered three different transfer plasmids, either containing a bi-directional dual promoter (BiPro), tandem promoter (TanPro), or a combination of a single promoter along with a cricket paralysis virus (CrPV) IRES. We performed an anti-sulfation ELISA on eCD4-Ig to determine the sulfation efficiency across the different cassette designs and observed no difference in sulfation efficiency, regardless of cassette design. We then assessed the potency of these variants through TZM-bl neutralization assays against several HIV-1 isolates. All newly engineered cassettes showed no decrease in potency against the selected HIV-1 isolates when compared to eCD4-Ig protein made from a two-vector approach. Next, we engineered six additional transgene cassettes utilizing the BiPro design. These expression cassettes include different promoters, stacked enhancers, and introns. When we assessed sulfation efficiency by ELISA, we observed higher efficiency for three of the five optimized BiPro designs. All newly engineered cassettes resulted in eCD4-Ig proteins with no decrease in potency or demonstrated improved potency against the selected HIV isolates when compared to eCD4-Ig protein from the first BiPro construct, suggesting that each protein was sufficiently sulfated for neutralization. Finally, we assessed eCD4-Ig expression in Balb/6 mice for two BiPro designs. Two cohorts of four mice were intramuscularly administered an AAV9 vector encoding one of the new BiPro designs. Our data show that eCD4-Ig expression varies between the two vectors with the best having a peak expression of 40 $\mu g/mL^{-1}$. Future mouse studies will focus on assessing expression of the remaining BiPro cassettes and further optimization will be made on the transfer cassette that results in the highest concentrations of eCD4-Ig.

1568 Adenovirus Type 34 and HVR1-Deleted Adenovirus Type 5 Do Not Bind to PF4: Clearing the Path towards Vectors without Thrombosis Risk

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Introduction: The adenoviral vector (Ad) based AstraZeneca and Janssen COVID vaccines have been associated with rare cases of vaccine-induced thrombotic thrombocytopenia (VITT, also known as TTS), a condition which depends on Ad binding to the blood protein Platelet Factor 4 (PF4). Harnessing the broad diversity of natural Ads, we strived to identify Ad types with low or absent affinity for PF4. Furthermore, we attempted to locate the binding site of PF4 on the capsid of the frequently vectorized Ad type 5 (Ad5) and construct modified vectors with ablated PF4 binding. Methods: We established an armamentarium of techniques including ELISA-qPCR and Aggregate Pull-Down (APD), which enabled fast and sensitive assessments of virus-protein interactions and could replicate already published results about PF4 binding of vaccine-like Ads. We used these techniques to screen dozens of types from various Ad species, and Ad type 5 (Ad5) derived vectors carrying genetic or chemical modifications of different hexon hyper-variable regions (HVR). The PF4 binding affinity of the main candidates was accurately measured by SPR. Results: Unlike most tested types, Ad34 did not bind to PF4. Likewise, the deletion or shielding through PEGylation of the HVR1 loop of Ad5 seemingly ablated its PF4 binding. We swapped the HVR1 loop of Ad5 and Ad34 to confirm that this region determines PF4 binding ability. Finally, we observed that interactions with PF4 increase Ad binding to erythrocytes and epithelium-derived cell lines, suggesting that PF4 may influence the tropism of vaccines in undesired ways. Conclusions: PF4 binds to Ad hexon through interactions dependent on HVR1. PF4 binding can be ablated by HVR1 deletion or exchange or through the vectorization of non-canonical Ad types. These findings open the way for the development of safer vectors for vaccine or gene therapy with decreased or suppressed risk of VITT.

1569 AAV Mediated Lysyl Oxidase Gene Therapy Improved Corneal Stromal Status: Application for Keratoconus

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Keratoconus (KC) is a progressive, corneal ectatic disorder caused by aberrant extracellular matrix (ECM) degeneration that reduces visual acuity and requires corneal transplant in severe forms. The disease primarily affects patients below age 40, estimated to affect 2.3% of people in India, causing significant morbidity. Lysyl oxidase (LOX), an endogenous collagen crosslinking enzyme is known to be reduced in the KC patient corneas, along with reduced collagens and increase ECM-degrading enzymes such as matrix metalloproteinases (MMP9) and inflammatory factors. Lack of collagens and crosslinking leads to corneal thinning and protrusion, hence current clinical interventions are dependent on surgical methods of stiffening the cornea or transplanting them, which are associated with many complications including a lack of donor corneal tissues. There is an unmet clinical need for efficient, long term, therapeutic strategies for treatment of KC. Enhancing LOX enzyme levels therefore can lead to efficient crosslinking of collagen fibrils, thereby lending stiffness and structural integrity in the cornea. We therefore developed a LOX expressing AAV vectors driven by CMV and Tetracycline responsive promoter which was further packaged in AAV9 capsids and investigated the effects of augmented LOX expression in corneal tissues using in vitro, in vivo and ex vivo models. We transduced primary human corneal fibroblast (HCF) from healthy and KC donors with AAV9-LOX. Immunoblotting was performed to assess the changes in the expression pattern of ECM proteins (LOX, type I and IV collagens, fibronectin, CTGF) and

MMP9. Increased levels of LOX led to the subsequent reduction in the MMP9 levels and a significant increase in various collagen proteins in both healthy and KC patient HCFs. We functionally validated this in a collagen gel contraction assay, which showed an increased contraction with media supernatants from cells transduced with AAV.LOX, indicating an increased level of collagen crosslinking by the secreted LOX protein. We further tested the effect of ectopic LOX expression from topically administered AAV9.LOX vectors in c57/bl6 mouse (N=6) corneas. Gene expression and immunofluorescence staining studies demonstrated that AAV.LOX transduction significantly decreased (4.5-fold) the MMP9 levels ($p< 0.01$) compared to AAV9.eGFP control transduced eyes. Similarly, collagen I and IV increased by 5.7-fold and 2.9-fold respectively compared to AAV9.eGFP control ($p< 0.01$). No ocular or corneal adverse events were noted in the mouse eyes treated with our vectors. Lastly, we carried out ex vivo studies by intrastromal injections of AAV9.LOX vectors in donor human whole eye globes and also transduced AAV9.LOX vectors in donor human corneal lenticules from SMILE surgeries (N=6). IHC analysis post intrastromal injection of AAV9.LOX virus showed a significant increase in LOX protein expression in the corneal stroma in comparison to the control eye upon histological analyses. An increase in collagen levels in the corneal stroma was also validated using Masson's trichrome staining. The human corneal lenticules recapitulated the same findings as the whole globe analysis. Taken together, the data indicate that augmentation of LOX via AAV-mediated gene therapy can improve the biomechanical stability of the cornea by enhancing the ECM content which has potential for clinical application.

1570 CD46 and CD59 Inhibitors Additively Enhance Complement-Dependent Cytotoxicity of Anti-CD38 mAbs Daratumumab and Isatuximab in Human Multiple Myeloma Cells

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Multiple myeloma (MM) is an incurable malignancy of the B-cell lineage, characterized by neoplastic, monoclonal expansion of plasma cells in the bone marrow. Remarkable progress has been made in the treatment of MM with the anti-CD38 monoclonal antibodies such as Daratumumab and Isatuximab, which can kill MM cells through the induction of complement-dependent cytotoxicity (CDC). The CDC efficacy of Daratumumab and Isatuximab is however limited by membrane complement inhibitors, including CD46 and CD59, which are upregulated in MM cells. We recently developed a small recombinant protein (Ad35K++) capable of blocking CD46 and sensitizing tumor cells to anti-CD20 mAb triggered CDC (e.g Rituximab and Ofatumumab). Here we tested Ad35K++ in combination with Daratumumab and Isatuximab. We show that Ad35K++ increases the CDC efficacy of Daratumumab and Isatuximab on Burkitt's lymphoma and MM cell lines (MOLP8, SUDHL-8, EJM). Ad35K++ salvaged the efficacy of Daratumumab and Isatuximab at subtherapeutic ("low") doses in MM lines. We also tested a small recombinant protein that targets CD59 (rILYd4) in combination with Daratumumab and Isatuximab on MM cells. rILYd4 also increased CDC killing of MM cells by Daratumumab and Isatuximab. The combination of Ad35K++ and rILYd4 additively enhanced the CDC effect of Daratumumab and Isatuximab. Daratumumab and Isatuximab treatment of MM lines (without Ad35K++ or rILYd4) resulted in the upregulation of CD46/CD59 and/or survival of $CD46^{high}$ / $CD59^{high}$ MM cells which escaped a second round of Daratumumab and Isatuximab treatment. Escape in the second treatment cycle was prevented by combining Daratumumab with Ad35K++ / rILYd4, whereby both co-therapeutics had an additive effect on Daratumumab0induced CDC. Studies with patient MM cells will be reported. Overall, our data demonstrate that Ad35K++ and rILYd4 are efficient co-therapeutics of Daratumumab and Isatuximab, specifically in multi-cycle treatment regimens, and could be used to improve the treatment of multiple myeloma.

1571 Optimization of Lentiviral Vector Mediated Transduction of T Cells for Clinical Use

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Genetic modification of T cells represents a straightforward technology to generate cell-therapy-based drugs, which showed promising clinical efficacy in cancer patients. Indeed, we demonstrated the feasibility of retroviral and lentiviral vector-genetically modified T cells production for commercial use as well as their clinical efficacy. Lentiviral vectors (LVV) are a platform to deliver and permanently express therapeutic genes and have displayed great potentiality in clinical trials. Here we present proof of concept results concerning the setting up of optimal T-cell transduction conditions with a highly-purified VSV-G pseudotyped LVV encoding for a CD44v6-CAR construct in order to obtain higher cell transduction efficiency and process yields. Briefly, donor-derived peripheral blood cells were activated with anti-CD3-anti-CD28 nanomatrix and cultured in chemically defined T-cell medium supplemented with interleukin-7 (IL-7) and interleukin-15 (IL-15). Different CD3/CD28-based activators and T cells specific serum free media were tested to define the best protocol for T cells activation and expansion. Transduction was performed after 24 hours or 48 hours, with MOI from 2 to 6 and cells were maintained in culture up to nine days. Optimized conditions were studied, Retronectin did not significantly improve the transduction rate of LVV. In addition, the small scale protocol was scaled-up in to a GMP-compliant environment . In particular, to this end, it was investigated the introduction of G-Rex platform for the cell expansion.

1572 Promoterless AAV Gene Targeting Vectors Favor Integration into the Albumin Locus Regardless of Homology Arm Sequences

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The GeneRide technology utilizes homology dependent integration of a promoterless AAV vector (AAV-HR) into the highly transcriptionally active *Albumin* locus, thus enabling sustained transgene expression in the growing liver. Transgene expression from on-target integrated

vectors is driven by the endogenous *Albumin* promoter generating a fusion transcript that is subsequently translated into the albumin protein and the transgene protein due to the incorporation of a P2A skipping sequence between the last coding exon of *Albumin* and the transgene. Here, we investigated AAV-HR mediated targeting to alternative genomic loci. We designed human Factor IX (huFIX) AAV-HR vectors with homology arms targeting the *Alb*, *ApoE* and *Gapdh* genes. Since a short sequence included in older versions of AAV ITR backbone vectors just upstream of the right ITR has been described to contain cryptic promoter activity (Logan et al., 2017), we cloned all constructs into an ITR backbone vector lacking this sequence. We used the original *Alb*-F9 vector (Barzel et al., 2015) that harbors the cryptic promoter sequence as a control (*Alb*-F9^{+c}). We also compared two *Albumin* AAV-HR vector designs, one with a 1.3kb left and a 1.4kb right homology arm identical to the original vector design (*Alb*-F9) and one with a shortened left homology arm (1kb) and a longer right arm (1.6kb), termed *Alb*^{m}-F9. B6 mice were infused with the different AAV-HR vectors and underwent partial hepatectomy 5 weeks post vector administration to reduce non-integrated episomal vectors. Plasma huFIX expression in the *ApoE*-F9 and the *Gap*-F9 groups was lower than in the *Alb*-F9 group. However, the group of mice that had been injected with the *Alb*^{m}-F9 vector showed higher expression levels initially followed by a drop at about 4 weeks. Due to the shortened left homology arm as compared to the original *Alb*-F9 vector a putative CTCF-only Cis regulatory element is absent in this vector design, possibly contributing to episomal expression. The *Alb*-F9^{+c} AAV-HR vector injected mice also showed a modest decline of initially high transgene expression following partial hepatectomy, thus confirming that the cryptic promoter sequence drives episomal expression. To evaluate if the AAV-HR vectors had integrated into the intended loci, mouse livers were harvested 12 weeks post vector administration and transcripts fused to the transgene on the 5' end were amplified using 5'RACE followed by high-throughput Oxford Nanopore sequencing. For each injection group 2-4 animals were analyzed in 5'RACE with a sequencing depth of 300,000 - 500,000 reads for each animal. We found that while the majority of 5'RACE transcripts obtained from mice injected with the AAV-HR *Albumin* targeting vectors were fused to the expected *Albumin* exons upstream of those encoded by the rAAV vector (Exons 13 and CDS of Exon 14), the other AAV-HR constructs resulted in transgene transcripts that were primarily fused to *Albumin* or other gene derived exons. We subsequently designed a huFIX AAV-HR vector containing random DNA instead of homology arms (RD-F9) as well as an AAV-HR vector with homology arms containing human *ALBUMIN* sequences *(huALB*-F9) and injected those constructs into mice. No transgene expression was observed in the mice that had received the RD-F9 AAV-HR construct while huFIX expression in the *huALB*-F9 injected mice was comparable to the levels seen in the *Alb*-F9 injected mice. 5' RACE as well as qRT PCR showed that a considerable number of transcripts were fused to *Albumin* exon 12 despite very low homology between human and mouse *Albumin* genomic sequences. These results suggest that *non-Albumin* AAV-HR vectors tend to integrate off target into the *Albumin* locus.

1573 Ribitol Supplementation Expands the Therapeutic Window of BET Inhibitor JQ1 for Triple Negative Breast Cancer

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Bromodomain and extraterminal inhibitors (BETi) are promising cancer therapies, yet prominent side effects have been reported in phase I clinical trials. JQ1 is the most thoroughly studied BETi that binds competitively to acetylated lysines and thus displaces BRD4 from binding to chromatin. JQ1 inhibits cancer cell proliferation, and this action involves downregulation of the rate-limiting enzymes of glycolysis, such as hexokinase 2, phosphofructokinase, and lactate dehydrogenase A. We recently reported that pentose sugar ribitol can upregulate glycosylation of alpha-dystroglycan (a-DG) with the potential to inhibit cancer cell proliferation and migration. Our following-up study by metabolomics revealed that ribitol can alter glycolysis in breast cancer cells. Here we investigate the combinatorial effects of ribitol and several other anticancer drugs including the bromodomain inhibitor JQ1 in breast cancer cells (MDA-MB-231, MCF-7 and T-47D) by cell survival assays and metabolomics. Different types of breast cancer cells were treated with each drug and in combination. All drugs alone showed limited inhibitory effect on cancer cells with the doses used. Combination of ribitol with the drugs such as chrysin, lonidamine, GSK2837808A, CB-839, and shikonin did not show additive effect. However, ribitol supplementation lowered the effective dose of JQ1 needed to successfully inhibit growth of the triple negative (MDA-MB-231) breast cancer cells compared to other types of breast cancer cells. Further, the combination of ribitol and JQ1 significantly inhibited the migration of triple negative breast cancer cells compared to either agent alone. Metabolomics and western blot data revealed that ribitol and JQ1 downregulate c-Myc levels leads to decreased activity of pyruvate kinase, glycolysis, and glutamine uptake. Further, the combination of ribitol and JQ1 increased the p53 levels leading to metabolic changes and apoptosis in the breast cancer cells. Altogether, these metabolic alterations of breast cancer cell mediated by ribitol and JQ1 result in a strong induction of apoptosis and inhibition of cell growth. Furthermore, ribitol supplementation might help reduce the severe side effects that arise from BETi therapy by reducing the dosage necessary for treatment. On the basis of our findings, further study is required to define biomarker(s) for targeted patient population for clinical trials with ribitol as an enhancer to BETi treatment. This study shows that metabolic reprogramming presents new avenues for developing targeted therapies to cancers with metabolites, especially in combination with other drug treatments and provides a possible clinical solution to challenges arising from the dose-dependent side effects of this class of epigenetic therapy.

1574 Early Changes in Liver Transcriptomic Profiles Following Adeno-Associated Viral Gene Therapy in the Severe Hemophilia A Dog Model

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Background: Recombinant adeno-associated virus vectors (rAAV) are a major gene therapy platform for treatment of monogenic disorders, including hemophilia A. Valoctocogene roxaparvavec (AAV5-HLP-hFVIII-SQ) is an AAV5 vector delivering B-domain-deleted (BDD) human FVIII transgene controlled by a hybrid liver-selective promoter. In a phase 3 trial, a single treatment of AAV5-HLP-hFVIII-SQ (6e13 vg/kg) provided therapeutic expression of FVIII and bleeding control in adult severe hemophilia A patients. However, the mechanistic basis of transaminitis, variability and durability observed in clinical trial participants are not clear. Understanding the molecular changes in the liver are critical to identify appropriate immune modulatory strategies for safety, efficacy, and long-term durability of AAV gene therapy. **Aim:** To investigate liver gene expression profiles before and after AAV5-HLP-canine-BDD-FVIII (cFVIII) administration in a severe hemophilia A dog model. **Methods:** Nine severe hemophilia A dogs received one of three vectors (non-codon-optimized AAV5-HLP-cFVIII-SQ, codon-optimized AAV5-HLP-cFVIII-SQ and AAV5-HLP-cFVIII-V3 at 6.0e13 - 2.0e14 vg/kg). Liver biopsies were collected at baseline and 3 months after vector administration. FVIII activity (FVIII:C) was measured by one-stage FVIII (OSA) assay using a pooled normal canine plasma standard. Liver cFVIII DNA and RNA levels were quantified using ddPCR. Transcriptomic profiling was performed by RNA-seq followed by pathway enrichment analysis. Cellular immune response in the peripheral blood mononuclear cells (PBMC) was evaluated using an IFN-γ ELISPOT assay. **Results:** Dose-related FVIII expression was observed in dogs treated with the codon-optimized vectors, with significant correlation between liver vector DNA and circulating FVIII:C (**Figure 1**). Dogs treated with codon-optimized cFVIII vectors demonstrated enhanced transgene expression compared to non-codon-optimized vector. No IFN-γ response was detected in PBMC. Transcriptomic profiling of liver biopsies demonstrated enrichment of integrin pathways, immunological gene signatures for B cells and plasmacytoid dendritic cells (pDCs), and common dendritic cells at 3 months compared to baseline (**Table 1**). Expression of inflammatory cytokines involved in NK-cell and T-cell activation were also enriched. **Conclusions:** Our data suggests that mild activation of B cells, dendritic cells, NK-cell, and T-cells with an inflammatory cytokine response occurred in the liver of AAV5-HLP-cFVIII treated dogs 3 months post gene transfer; albeit without transaminitis. Transcriptomic profiling of PBMCs is ongoing to compare to liver profiles to better understand kinetics of immune responses to rAAV.

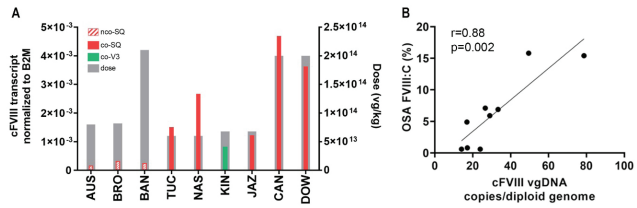

Figure 1. AAV5-cFVIII expression and activity in severe hemophilia A dogs at 3 months. A) AAV5-cFVIII mRNA transcript levels, and B) correlation between liver vector DNA and OSA FVIII:C, one-stage FVIII clotting assay (canine normal pooled standard) with a lower limit of detection of 2%. co, codon optimized; nco, non-codon optimized; SQ and V3, B-domain-deleted FVIII variants.

Enriched Gene Set	p-value	FDR-adjusted p-value	No. of genes in term	No. of genes that were regulated following AAV5-HLP-cFVIII treatment	No. of up-regulated genes	No. of down-regulated genes	Normalized Gene Set Enrichment Score (NES)
Integrin pathway	0.00058	0.034	22	3	3	0	1.47
B cells and plasmacytoid dendritic cells	3.40E-06	0.014	168	10	9	1	1.80
Common dendritic cells	1.00E-05	0.020	187	10	8	2	1.49
Cytokine production	7.00E-06	0.015	668	19	16	3	1.56

Table 1: Gene set enrichment analysis comparing AAV5-HLP-cFVIII vectors administered to hemophilia A dogs at 3 months and baseline (treatment vs control). A normalized gene set enrichment score (NES) determines whether a gene set is positively regulated or negatively regulated and an NES with Benjamini-Hochberg adjusted *P* value <0.05 was considered statistically significant. FDR, False discovery rate.

1575 Intensified Enveloped Vector Production Using Continuous Perfusion Bioprocessing

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Background A great demand of vectors for gene therapy and vaccination drives development of disruptive technologies for vector production beyond established technologies for non-enveloped gene transfer vectors such as AAV. New modalities in the cell and gene therapy industry also require innovation in process technologies and manufacturing methods to keep pace. We address this need by evaluating tangential flow depth filtration (TFDF) technology for the perfusion process to produce enveloped vectors from a human-derived cell line in single use 3L bioreactors. Although alternative tangential flow (ATF) and tangential flow filtration (TFF) have been applied to the perfusion process for producing monoclonal antibodies due to their large membrane surface, ease to implementation, high recovery of viable cells, and a small footprint, fouling of the surface of ATF and TFF fibers associated with the build-up of cells, cell debris, particulates and the extracellular secretive products are one of the challenging issues for a long perfusion process. TFDF uses a depth filter with a micro-meter pore size in a tangential flow mode, realizing a high efficiency in cell retention and circumventing the fouling issue. **Methods and Results** The human-derived suspension cells were expanded in shake flasks prior to inoculation into 3L single use bioreactors. The inoculation density was in the range of 1-5 x 10^6 cells/mL. The bioreactors were

controlled via BIOSTAT-DCU II at a power per unit volume of 10-30 W/m3, a temperature of 37°C, a pH set point of 7.0 and a dissolved oxygen concentration of 40%. TFDF filters (30 cm^2) at a pore size of 3-5 micron were used for the perfusion process, and the shear rate cross the filter surface was kept at 2000 s-1. Various permeate flow rates in the range of 0.5- 5.0 VVDs (volume per vessel volume per day) were applied. The viable cell density of 50- 60 x 10^6 cells/mL was achieved after employing a cell specific perfusion rate (CSPR) in the range of 40-60 pL/cell/day and manual or continuous cell bleed, and the cell viability was greater than 80% after more than 10 days of perfusion. The physical and infectious titers of the vectors produced in the reactors were very similar to those in the permeate, thus suggesting that the sieving efficiency of the TFDF filters for these enveloped vectors was around 100%. The TFDF filter allowed us to maintain the production culture up to 10 days without filter fouling, and we could continue to collect the vectors produced each day, enabling 10-20 fold greater total vector production. **Conclusion** Our study demonstrates the feasibility of applying TFDF filters in the perfusion process for the production of an enveloped gene transfer vector. The TFDF technology could have a great potential in the perfusion process for producing various therapeutic products from mammalian cells.

1576 HLCN061: An "Off-the-Shelf" Gene Engineered Human iPSC-Derived NK Cell Product for the Treatment of Solid Tumors

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NK cells are a member of innate immunity and contribute on elimination of virus-infected cells, stressed cells, and tumor cells. Different from T cells, NK cells do not need to be sensitized before target and do not cause GVHD in the allogenic setting. Thus, NK cells are thought to be a good candidate of off-the-shelf products for adoptive cell therapy. Currently, peripheral blood or cord blood has been used for the major source of NK cells for immunotherapy. However, these NK cells are heterogenous, and the efficacy of these cells is donor dependent. While iPSC-derived NK cells are easy to control their quality by standardizing the starting material. Moreover, iPSC has an advantage in gene modification to generate a desired cell. We developed HLCN061, NK cells derived from genetically engineered human iPSC (hiPSC), expressing molecules that enhance the anti-tumor activity, homing to tumor and persistence for the treatment of solid tumors. *NKG2D*, high affinity *CD16*, *CCL19*, *CCR2B* and *IL-15* genes under the control of the human *EF1a* promoter were introduced into hiPSCs by using PiggyBac transposase system. Then we selected a genetically engineered hiPSC clone 18H5 by expression of transgenes and NK lineage differentiation capacity. The clone 18H5 showed a normal karyotype, and insertion of exogeneous genes shows minimum effect on expression of endogenous genes nearby insertion sites of integrated genes. Furthermore, we developed a robust manufacturing protocol for differentiating hiPSCs to NK cells using an automated perfusion feeder-free culturing system. Our system can manufacture 5x10^{10} cells of HLCN061 from 1x10^5 cells of clone 18H5 by using a 3-liter bioreactor. HLCN061 showed robust anti-tumor activity against various cell lines derived from solid tumors. Interestingly, primary NK cells showed a reduction of cytotoxicity against IFN-γ-treated tumor cells, while HLCN061 showed higher cytotoxicity against them. HLCN061 was able to migrate to the inside of tumor spheroids dependent on its CCR2B expression. Forced expression of IL-15 enhanced the persistence of HLCN061 in immunodeficient mice without the support of human cytokines. Intravenously injected HLCN061 was still detected in the lung at 39 weeks after injection without tumorigenesis. Finally, HLCN061 did not show any cytotoxicity against normal human PBMC. Thus, these in vitro and in vivo proof-of-concept studies promise that HLCN061 might be a good adoptive cell therapy product for the treatment of solid tumors.

1578 Rare Disease Patient Advocacy Perspectives on the Promise and Challenges of Gene Therapy

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Background: Gene therapy is a rapidly evolving field that offers new hope while presenting unique challenges. It is vital to understand the questions, expectations, and fears of patient communities and to share learnings across diseases. This approach can help to ensure community perspectives are incorporated into the development of potential treatments. An advisory council was formed to meet with patient advocacy group (PAG) leaders to better understand a range of perspectives on gene therapy interest, knowledge, and acceptance and identify shared goals in developing and providing gene therapy information. **Methods:** Four hybrid meetings were held with 9 PAG representatives in five countries across a range of rare diseases. Meetings also included a moderator and biotechnology employees. Meetings lasted 11 hours over 4 months. Pre-meeting surveys were conducted, containing open-ended and discrete questions to help lead discussion. Discussions were recorded to aid in meeting summarization. **Results:** In general, the communities represented were curious and enthusiastic about gene therapy, though the same factors driving acceptance were also factors behind hesitancy. Information and education gaps can lead some community members to underestimate risks and overestimate benefits associated with gene therapy, and others to avoid gene therapy all together. Gene therapy is sometimes seen as a "cure" and a "silver bullet," with enthusiasm often motivated by a desire to have lasting relief from daily management of conditions and experience a sense of predictability, normalcy, and freedom. Simultaneously, lack of knowledge and uncertainty of the extent and durability of benefit

lead others to view gene therapy as an unacceptable risk. Clear and plain-language education on basic gene therapy facts can help promote understanding and demystify the topic and current research. Managing expectations and clearly communicating benefits and risks of gene therapy are critical and require different approaches at each stage of development and commercialization. Healthcare professionals are trusted partners in gene therapy decision making; however, not all patients have access to knowledgeable specialists. Patients treated with gene therapy also have a strong influence and can help address myths and misinformation by sharing their lived experiences, especially in rare disease. PAG leaders expect biopharma companies studying gene therapy to partner with communities to provide transparent information and help manage expectations at all stages. **Conclusions:** Bringing together diverse PAGs and gene therapy specialists identified core themes that need to be addressed to support gene therapy decision making in communities where it is being actively studied. These include 1) supporting growth and building capacity of PAGs and 2) developing different education approaches to manage expectations and demystify gene therapy in general and for specific disease states. Biopharma companies, healthcare professionals, advocates, and patients have critical roles to play in providing information. Additional research should explore how information-seeking practices of patient communities may shape the understanding of and interest in gene therapy.

1579 Generation and Evaluation of rAAV Vector for the Treatment of COX20 Deficiency

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Cytochrome c oxidase 20 (COX20) is a ubiquitously expressed protein. It plays vital roles in assembling mitochondrial complex IV by stabilizing its COX2 subunit on the mitochondrial inner membrane as a chaperone protein. Loss of COX20 leads to reduced protein level and enzyme activity of complex IV. COX20 deficiency is a rare, autosomal recessive disease, although increased cases have been reported in the last few years. Patients with COX20 deficiency develop early onset hypotonia, ataxia, areflexia, dystonia, dysarthria, and sensory neuronopathy but without severe cognitive or intellectual disabilities, suggesting defects in the peripheral nervous system and a primary degeneration of sensory neurons in the dorsal root ganglia. Unfortunately, the current treatment of COX20 deficiency is limited to supportive care, and effective therapy is still unavailable. In this study, we developed recombinant adeno-associated virus (rAAV)-based gene therapy vectors for the treatment of COX20 deficiency. Two isoforms of COX20 (201 and 203) exist, but their therapeutic implications are unknown. To differentiate between endogenous and exogenous COX20, we designed six vectors with either isoform and with or without FLAG-tagged to the c- or t-terminus driven by the chicken beta-actin (CB) promoter. Protein expression analysis confirmed that protein levels were equivalent for COX20 with or without FLAG tag. The mitochondrial localization of COX20 is essential for its function. Therefore, we first evaluated the different constructs' ability to express COX20 that localizes to mitochondria. Using a co-localization assay, we found that COX20 expressed from each construct localizes to mitochondria. Subsequently, all constructs were packaged into rAAV9 to evaluate their function and possible toxicity in mice via systemic delivery. In summary, this early proof-of-concept study holds promise for the development of gene therapy for COX20 deficiency.

1580 Development of Adeno-Associated Virus Producer Cell Lines by Small Molecule Inducible Expression of Packaging Genes

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A major challenge in the use of recombinant adeno-associated viruses (rAAVs) for gene therapy is the requirement for large scale production of the viral particles. A traditional method for vector production is the triple transfection method, where packaging (Rep and Cap), adenovirus helper genes, and transgene elements on separate plasmids are transiently expressed in production cell lines such as HEK293T. A major limitation of this method is the requirement that each cell receives all three plasmids to produce rAAV, reducing the potential efficiency of viral production. One strategy to overcome this limitation is the generation of production cell lines that stably express one or more of the packaging genes and thereby reduce the number of plasmids delivered by transient transfection. However, constitutive expression of Rep is toxic in many cell lines, limiting the utility of this approach. To overcome this limitation and generate producer cell lines that do not require the triple transfection mechanism, we designed a Rep expression construct that expresses Rep protein only upon treatment with a small molecule. Here, we demonstrate that transient transfection of this construct and the other packaging components allows for inducible Rep expression and results in packaging of rAAV following small molecule administration. We then generated stable cell lines that express this construct and demonstrated Rep inducibility and AAV packaging. These cells will provide the opportunity for increased vector production efficiency, and potentially decrease production costs and increase scalability.

1581 Development of Allogeneic HSC-Engineered iNKT Cells for Off-the-Shelf Cancer Immunotherapy

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Cell-based immunotherapy has become the new-generation cancer medicine, and "off-the-shelf" cell products that can be manufactured at large scale and distributed readily to treat patients are necessary. Invariant natural killer T (iNKT) cells are ideal cell carriers for developing allogeneic cell therapy because they are powerful immune cells targeting cancers without graft-versus-host disease (GvHD) risk. However, healthy donor blood contains extremely low numbers of endogenous iNKT cells. Here, by combining hematopoietic stem cell (HSC) gene engineering and in vitro differentiation, we generate human allogeneic HSC-engineered iNKT (AlloHSC-iNKT) cells at high yield and purity; these cells closely resemble endogenous iNKT cells, effectively target tumor cells using multiple mechanisms, and exhibit high safety and low immunogenicity. These cells can be further engineered with chimeric antigen receptor (CAR) to enhance tumor

targeting or/and gene edited to ablate surface human leukocyte antigen (HLA) molecules and further reduce immunogenicity. Collectively, these preclinical studies demonstrate the feasibility and cancer therapy potential of AlloHSC-iNKT cell products and lay a foundation for their translational and clinical development.

1582 Exploration of Immune Cell and Cytokine Biomarkers after Multiple Administrations of Autologous Natural Killer Cells in Patients with Solid Tumor

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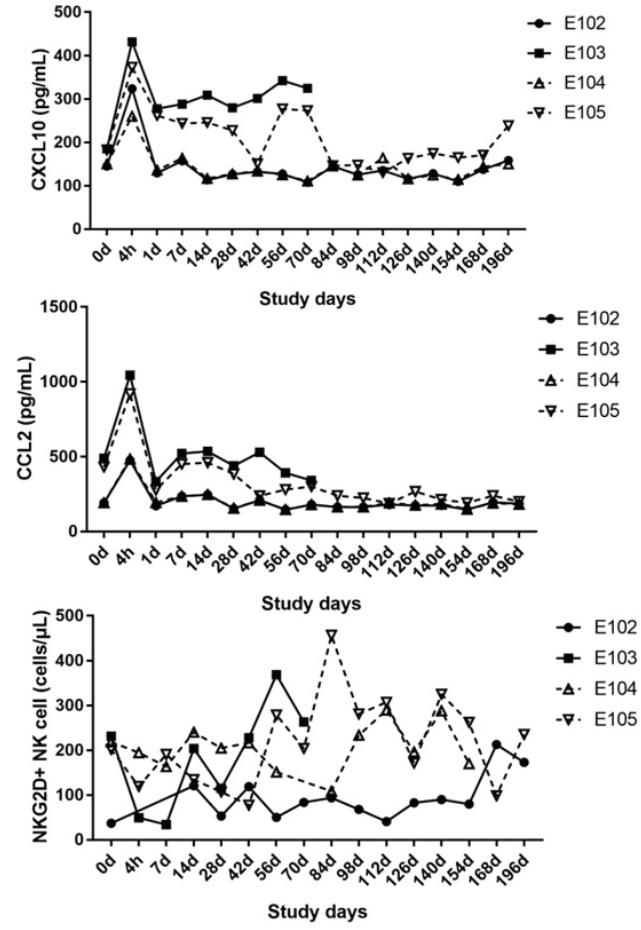
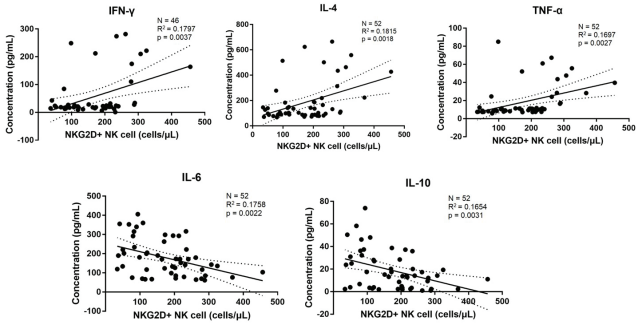
Natural killer (NK) cells have been studied widely due to its immunotherapeutic potential for various indications. To develop NK cell therapy for clinical implications successfully, the basic data should ground to establish the standard regimen based on biomarkers to monitor safety and exposure-response relationship. From these perspectives, the most of previously reported studies have limitations such as partial or short-term observation of biomarkers, resulting in difficulties of comprehensive interpretation. Thus, we aimed to explore the biomarkers including various types of immune cells (NK cell, T cells, and myeloid-derived suppressor cells) and cytokines (tumor suppressor and protumoral cytokines) and their relationships after multiple administration of CBT101 (autologous NK cells) in patients who underwent curative surgery and adjuvant therapy for solid cancer. The patients received the CBT101 intravenously 13 times every 2-weeks. Whole blood samples for cell and cytokine biomarkers were obtained up to 4 hours after the first administration and before each subsequent administration. Four patients were enrolled and three patients completed the study. The cytokines including CXCL10 and CCL2 increased at 4 hours after first administration. The trough values of NKG2D$^+$ NK cell counts measured before each administration showed steady increase until 5-6 administrations. NKG2D$^+$ NK cell counts presented positive correlations with tumor suppressor cytokines such as IFN-γ, TNF-α, and IL-4 and negative correlations with tumor growth cytokines such as IL-10 and IL-6. The findings of this study suggested the needs for reference values of the biomarkers and for further studies with larger sample size to understand of mechanism of action of NK cells and to form the basis for clinical implications.





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1584 Upregulation of AEG-1 Expression Attenuates Granule Cell Dispersion and Seizure Development by Suppressing mTORC1 Activation in KA-Induced TLE Mice

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Although granule cell dispersion (GCD) in the hippocampus is known to be an important feature associated with epileptic seizures in temporal lobe epilepsy (TLE), the endogenous molecules that regulate GCD remain elusive. Here, we have identified that the expression of AEG-1 is upregulated in the DG of a KA-induced mouse model of TLE. We further demonstrated that AEG-1 upregulation by AAV1 delivery in the DG induced anticonvulsant activities such as the delay of seizure onset and inhibition of spontaneous recurrent seizures (SRS) through GCD suppression in the mouse model of TLE, while the inhibition of AEG-1 expression increased susceptibility to seizures. The present observations suggest that AEG-1 is a novel regulator of GCD formation and seizure development associated with TLE, and the induction of AEG-1 in the DG may have a therapeutic potential against epilepsy.

1585 Immunodeficient Mouse Models to Assess Human HSPC Engraftment and Gene Editing: NSG vs. NBSGW

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Humanized mouse models are an essential tool in the field of gene therapy to assess long-term engraftment of human hematopoietic stem and progenitor cells (HSPC) and persistence of their genome editing. In the NSG (NOD-Prkdc^{scid}, IL2rg^{null}) strain, the combination of the NOD, SCID (DNA-PK) and IL2rg mutations results in an ablation of adaptative immunity, severe deficiency of innate immunity and a high receptivity to the engraftment of human HSPC after cytoreductive irradiation. Introduction of the viable c-kit mutant gene (Kit$^{W41/W41}$) into the NSG strain resulted in a new mouse model (NBSGW) in which transplanted human HSPC have a competitive engraftment advantage, yielding enduring engraftment without irradiation. We hypothesize that the facilitated engraftment of human HSPC due to the genetic disadvantage of host NBSGW mice may allow the engraftment of more mature, less primitive CD34+ progenitor cells, diminishing the ability to distinguish adverse effects of *ex vivo* manipulation on long-term primitive HSPC engraftment potential. The consequences of this are that the toxicity derived from gene editing protocols might be underestimated, while hematopoietic stem cell potential and long-term maintenance of genome-editing might be overestimated. To test this hypothesis, NSG and NBSGW mice were transplanted from the same pool of mobilized peripheral blood HSPC edited using CRISPR/Cas9 to correct the *HBB* sickle mutation by a single stranded deoxyoligonucleotide donor. Both mouse models received the same number of cells, and while the NSG mice (n=12) had sub-myeloablative irradiation (250 rad), the NBSGW mice (n=13) did not. Four months after transplant, mice were euthanized and human cell engraftment and *HBB* gene correction were assessed in the bone marrow (BM) and spleen of the mice. No deaths occurred in the NSG group from the conditioning irradiation. Human CD45+ cell engraftment was about 1.5-times higher in BM and spleens from the NBSGW mice than in the NSG mice. The same hematopoietic cell lineage distributions were observed in BM and spleen in both mouse models. Gene correction by homologous directed repair (HDR) and non-homology end joining (NHEJ) were significantly higher in the NBSGW (HDR- 22±2.7%, NHEJ- 52.5±4%) than in the NSG mice (HDR- 18.4±1.1%, NHEJ- 56.7±2.4%) in BM, but no differences were found in spleens. Human CD34+ cells and murine CD45+/lineage-negative cells were sorted from the BM from 6 mice from each group. Single cell-RNA-sequencing (sc-RNA-Seq) is ongoing to characterize any transcriptional differences in the human HSPC engrafted in each mouse model, as well as to investigate the phenotype of the murine HSPC from each model. Secondary transplants were performed with BM from 5 NBSGW and 5 NSG primary recipients transplanted into equal numbers of both NBSGW and NSG secondary recipients. No significant differences were observed between the two mouse models in either secondary engraftment of human CD45+ cells (ranging from zero to 2%) or gene editing (with marking detected in all the engrafted mice but one mouse). We conclude that the NBSGW mice may allow persistence of more committed and proliferative progenitor cells, resulting in significantly greater percentages of human cells. NBSGW xenografts may show higher HDR frequencies because the committed progenitors are less quiescence and more able to correct by HDR the double strand breaks produced by Cas9 nuclease, compared to the more quiescent less committed HSPC. As such, the NSG model may provide a more stringent assessment of long-term engrafting HSPC and HDR frequency. It is important to be understand differences in model systems to use them to most accurately predict outcomes of clinical gene therapy.

1586 Achieving Uniformity in Transduction with AAV Gene Therapy to Muscular Dystrophy

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AAV-mediated gene replacement therapy is currently the most promising treatment for muscular dystrophies (MD). Recent clinical trials have shown the potential of the therapy to delay and even cure the diseases. However, multiple side effects, including renal toxicity, liver damage up to fatalities have been reported by ongoing clinical trials. Importantly, severe incidences are clearly dose-related, with fatalities largely linked to AAV dosage above 1e14 vg/kg body weight. Therefore, achieving efficacy with lower AAV dose is critical for AAV gene therapy to MD as discussed recently by Cellular, Tissue, and Gene Therapies Advisory Committee (2021). Toxicity in clinical applications is convoluted with another concern, the heterogeneity of transgene expression when the dose of AAV is lower than the potential lethal doses. Preclinical animal model tests and reports from clinical trials with doses lower than 1E14 vg/kg induce variable transgene expression between muscles and among fibers of individual muscle including heart

and diaphragm. There is little doubt that the fibers lacking transgene expression will continue to degenerate and transgene overexpression could also be detrimental. AAV gene therapy therefore faces a dilemma of either using super high doses of currently available AAV serotypes and vector systems to achieve desirable efficacy with high risk of severe side effects, or opting to limit AAV dosage for safety, but achieving suboptimal and probably shorter-term efficacy. Two main approaches can be used to dissolve the dilemma. One is to explore new and more potent AAV strains with higher tropism to skeletal and cardiac muscles. Several recent reports have shown propitious progress with the development of MyoAAVs and AAV.cc47 which showed superior efficiency and selectivity to transduce muscles in mice and non-human primates. However, these synthetic strains of AAV produce the same variable distribution (mosaic) pattern in muscles, containing fibers with transgene expression ranging from higher than normal to undetectable within individual skeletal and cardiac muscles. While viral tropism plays role for variable expression, differential activation of promoters by different muscles and fiber types is also a critical factor. Therefore, one alternative solution to minimize heterogeneity is promoter modulation. This has been extensively studied in the early stage of AAV gene therapy to muscular dystrophy, but with focus on increasing efficiency rather than distribution in transduction. All promoters currently in use produce highly variable expression. In this study, we have constructed several synthetic regulatory cassettes based on muscle specific MCK promoter/enhancer components in combination with other essential regulatory elements with the aim to improve transduction efficiency and especially distribution in cardiac and skeletal muscles. The expression vectors with the cassettes were packed by AAV9 and the virus was delivered systemically in a FKRP mutant mouse models with P448L mutation. The mice experience progressive dystrophic phenotype with extensive muscle degeneration and shortened life span. One of the regulatory cassettes MCKopt5 produces higher levels of transduction in both cardiac and skeletal muscles than CK7 driven vector. More importantly, FKRP expression restored matriglycan expression homogeneously in both types of muscles when only 4e12vg/kg was administrated whereas CK7 based promoter requires 5e13vg/kg to achieve expression in near 100% muscle fibers of skeletal muscles. This pattern of transduction is highly desirable for achieving long-term efficacy with low AAV dosage, avoiding dose-related toxicity. A combination of the synthetic regulatory cassette with high muscle tropism AAV serotype constitute ideal AAV gene therapy to muscular dystrophy.

1587 Exploiting a Novel Gene Regulatory Code for Retinal Gene Therapy

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Rhodopsin-Autosomal dominant Retinitis Pigmentosa (RHO-ADRP) is a progressive photoreceptor degeneration leading to an incurable loss of vision. To treat this blinding inherited disease, we established a solid gene therapy proof of principle with Adeno-associated virus (AAV) vectors, which couples two approaches, gene silencing with gene replacement. Silencing and replacement approach was generated by inserting in a single AAV vector two expression cassettes carrying independent promoter elements of differential strength as drivers for the silencer and the replacement components. The silencing step is achieved through an unconventional transcriptional repression system, in which an exclusive use of DNA-binding domain (DBD) (ZF) operates without the aid of repressor domains. We demonstrated that the sole binding of this ZF-DBD (ZF6) to a short and accessible (unbound from endogenous transcriptional modulators) DNA Cis Regulatory Elements (CREs) blocks RHO expression, at low doses with both limited off targeting and engagement of cellular systems. Based on this result, we further explored these CREs with higher resolution by changing the number, orientation, order, spacing and their sequence composition demonstrating a wide range of modulation of the transcriptional outputs. By exploiting the gene regulatory code found, we obtained a reciprocally controlled transgenes expression of silencing and replacement which also operates in a wide range of vector doses. Hence, a safer and more efficacious gene therapy of RHO-ADRP is demonstrated.

1588 A Novel Next-Gen Sequencing Library Preparation Method to Sequence Small, Long, and Low-Quality (Fragmented/Degraded) RNA with Low (*Pico*-Grams) Quantity of Templates for Better Diagnostics, Biomarker Discovery, and Research

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Background and limitations of current technologies*:** Next-generation sequencing (NGS) of RNA and DNA is a method of high-throughput sequencing of biological samples. There are several well-established methods in published literature and also commercially available in the form of products/kits dedicated to sequencing total RNA, DNA, exome, small RNAs, and selected panels of gene/ loci to address specific issues.However, it has been a great challenge to sequence highly fragmented and degraded DNA/RNA due to the limitations of library preparation methods. This affects the sequencing of clinical samples, biological research materials, liquid biopsy, FFPE tissue, exosomes, forensics, cell-free RNA/DNA, and archeology, among several other types of materials. A large fraction of archived and even fresh samples do not generate high-quality data, which cripples basic research, biomarker discovery, and diagnostics. Currently, it is also impossible to sequence small RNAs alone or small and long RNAs together with pico-gram quantities of total RNA template. ***Methods and results: We have addressed these limitations by developing a novel method to prepare NGS libraries from pico-gram quantities of total RNA templates. Our methods could efficiently capture RNA of all sizes (20bp and above, including small and long RNAs) simultaneously and detect 2-10 times more unique transcripts at a similar sequencing depth than the current state-of-the-art methods. We could capture 20,000 to 100,000 more transcripts than an established method in a proof-of-concept study. We could capture small (as small as 20 bases and above) and long RNAs in the same library using very low (pico-grams) quantities of total RNA. ***Applications and broader impacts:*** Our method is applicable to highly fragmented and degraded samples like biological samples,

clinical samples, FFPE tissue, cell-free RNA/DNA, liquid biopsies, archeological samples, and also normal samples. The variations and errors observed in NGS data due to differential degradation between samples are negligible in our method. This helps generate consistent and high-quality sequencing data. We could generate 2-10 times more high-quality data with as little as 500pg of total RNA, which is close to the total RNA obtained by a single cell and hence applies to single-cell sequencing. The method is compatible with automation as well. Due to its high efficiency and sensitivity, we think our method will enable better diagnostics, biomarker discovery, and research.

1589 Deep Characterization of Allogeneic PBMC Donor Starting Materials to Assure Better Quality Cell Immunotherapies

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The increased focus on the development of allogeneic cell immunotherapies for the treatment of cancer has highlighted the need for deeper characterization of donor starting materials, in particular PBMCs that may serve as source material for various adoptive immunotherapy approaches. We report on our efforts in this aspect by evaluation of healthy donor immune cell repertoires by FACs, including CD3, CD4, CD8, CD14, CD19, CD45 and CD56 cell subsets present in leukapheresis samples collected from donors ranging in age from 18 to 70 years. Additionally, we have characterized PBMC, CD3, CD4 and CD8 cell subsets for functional aspects that are key aspects of potency and durability of response in cancer indications. These include their effector function potential using a proteomics-based assay platform, metabolic fitness as reflected in glycolytic rates, and standard cytokine release and cell killing assays. To date the results indicate significant donor variability in all the parameters evaluated and a clear correlation to donor age in terms of effector function, metabolic state, and specific immune cell subset levels. These data indicate that deeper donor starting material characterizations may facilitate better cell immunotherapies in terms of potency and effectiveness for the treatment of oncology indications.

1590 An Electroporation Platform for Enhanced Non-Viral Genome Editing with Cutting-Edge Buffer for Optimal Homology-Direct Repair

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Transfection of cells by introducing foreign nucleic acids or proteins to alter their genotype or phenotype is crucial in a variety of life science applications. A wide range of transfection methods exists and choosing which approach to use often depends on its suitability to the application in question. Electroporation is a physical transfection strategy that uses an electrical pulse to create temporary pores in cell membranes through which genetic materials can pass into cells. It is a highly efficient and powerful tool that has been shown to have superior performance with gene editing-based payloads, such as CRISPR and TALEN. The Invitrogen Neon Transfection System, a widely used research-focused electroporation instrument, has been shown to be a reliable electroporation tool. It utilizes a pipette tip chamber to generate a uniform electric field for a significant increase in transfection efficiency and cell viability. Neon NxT Electroporation System is an improved electroporation instrument inspired by the Neon System and maintains the "tip" electroporation design. It leverages key competitive advantages secured by the Neon. We have observed a consistent equivalent or better performance of the Neon NxT Electroporation System when compared to its legacy product Neon Transfection System using a wide variety of mammalian cells and payloads. We have observed 80 to 90% transfection efficiency for GFP plasmid DNA in HeLa, BC-1, Jurkat, and HEK293 using both 10 µl and 100 µl reaction tips of the Neon NxT Electroporation System. For eGFP mRNA, we observed more than 90% transfection efficiency in Jurkat, HEK293, and Primary Naïve T cells. In terms of CRISPR/Cas9 regulated HDR-driven non-viral genome editing, we have observed 40-70% knock-in efficiency in Jurkat, K562, and primary activated T cells. We have observed more than 90% CRISPR/Cas9 regulated knock-out efficiency in primary activated T cells and hematopoietic stem cells (HSCs). In addition, we have observed more than 50% CRISPR/Cas9 regulated knock-out efficiency in primary natural killer (NK) cells using Neon NxT Electroporation System. Electroporation of these cells had minimal effect on cellular viability. In Addition, the Neon NxT Electroporation System introduces Resuspension Genome Editing Buffer. The Neon NxT Electroporation System in conjunction with Resuspension Genome Editing Buffer is a powerful tool used to increase knock-in efficiency in mammalian cells through CRISPR/Cas9 genome editing. It was designed to drive the rate of homology-directed repair (HDR) in cells. The system has been demonstrated to significantly increase knock-in efficiency in three different mammalian cell types, with an approximately 5-fold increase in Jurkat cells, a 2-3.7-fold increase in K562, and activated primary T cells, respectively. The system also improved knockout/cleavage efficiency in hematopoietic stem cells (HSCs) and natural killer cells (NKs). Overall, the Neon NxT system provides broad utility in CRISPR/Cas9 applications by improving HDR-derived knock-in efficiency in a range of mammalian cells.

1591 The Need for Speed: Maximising Adeno-Associated Virus (AAV) Vector Productivity with High Throughput (HTP) Platforms

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Pharmaron has established a multi-faceted purification toolbox approach using small scale upstream processing (USP) and downstream processing (DSP) HTP systems, to deliver rapid process optimisation and manufacture of a wide range of Adeno-Associated Virus (AAV) serotypes and products. With utilisation of Ambr15 and Ambr250 bioreactor systems, Pharmaron can provide an initial small-scale assessment of a product's performance in their platform process at a fraction of the cost, space and resource of a full lab scale study. Equally the use of an automated liquid handling robot in DSP, allows Pharmaron to conduct an early assessment of purification for a gene therapy product. They can then concentrate on a targeted HTP screening approach utilising DoE to assess, for example, varying

seeding densities and transfection conditions in USP, to varying chromatography buffers and resins in DSP. This targeted approach drastically reduces chemistry, manufacturing and controls (CMC) development timelines, maximises productivity and drives results. Despite this, the use of HTP and automated processes can cause bottlenecks in an end-to-end platform which delivers gold standard analytics as an end point; therefore, Pharmaron has developed a number of HTP analytical methods to match this output. Through integration of size exclusion (SEC) and anion exchange (AEX) ultra-performance liquid chromatography (UPLC) systems for fast titre generation and Empty:Eull capsid quantification, Pharmaron has developed a testing strategy for rapid screening of hundreds of samples in a 24-hour period. Here they show that high throughput platforms provide advanced technical solutions which drive reproducibility, productivity and reduced process times.

1592 Azide-Functionalized SpCas9 Enables Regioselective Conjugation of Auxiliary Therapeutic Molecules Such as Functional siRNA

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CRISPR gene therapy is reaching adolescence, as the first landmark clinical trials are underway. Current *in vivo* gene editing focusses on functional gene knock-out, in which the Cas9 protein is used to create double stranded DNA breaks. Following this, DNA repair by non-homologous end-joining induces frameshift mutations, knocking out the gene. Other CRISPR apoplications such as faithful gene correction are still an actively developing field. Progress towards improving gene correction was made by engineering the SpCas9 protein to exploit novel gene correction modalities. Most examples are fusion proteins, such as base-editors and prime-editors. The design space for SpCas9 protein fusion is however limited to the C-terminus due to the presence of active sites near the N-terminus. This limitation prompted the current study, in which we aimed to develop azide-functionalized SpCas9 for click-chemistry to the protein surface. The main aim was retaining protein activity following conjugation of functional molecules to SpCas9.First we identified regions on the protein surface in which SpCas9 can be modified without loss of activity, based on earlier literature. We chose to substitute exposed aromatic amino acids in these regions based on the SpCas9 crystal structure (PDB: 4cmp) with p-azido-phenylalanine (pAzF) as click-chemistry handle. pAzF was introduced by recombinant expression in *E.coli* co-expressing pEVOL-pAzF (Addgene #31186) for amber STOP codon reprogramming. After purification we characterized four candidate pAzF-SpCas9 constructs on their bioactivity, purity and conjugation capabilities to Alexa Fluor 647 (AF647)-DBCO. One variant in particular, 539pAzF-SpCas9, retained bioactivity after conjugation. We applied this 539pAzf-SpCas9 platform for co-delivery of SpCas9 RNP complexes and siRNA as model for synergistic therapy. We functionalized siRNA with a releasable linker containing TMTHSI for copper-free click conjugation to the SpCas9 protein. The linker releases its cargo in reducing environments such as the cytosol. This results in release of unmodified siRNA and SpCas9 with a small chemical side group on the azide. In a proof of concept study we show that SpCas9-siRNA complexes are generated with a 50-90% conjugation efficiency after two hours based on gel electrophoresis and densitometry. The conjugation is furthermore reversed in presence of 5 mM glutathione. Furthermore we show that in model cell lines for Cas9 and luciferase siRNA activity (HEK293t expressing eGFP or dual luciferase, respectively) both components are active after lipid nanoparticle delivery. Finally we show that co-encapsulation and co-delivery is improved by conjugation, based on differential fluorescent labeling (AF647SpCas9-AzF and rhodamine 3b-siRNA). We conclude that SpCas9 and additional therapeutic molecules is possible by regioselective conjugation, without significant activity loss of either cargo molecule. We intend to follow up with biologically relevant siRNA to improve CRIPSR gene correction using the homology-directed repair pathway.

1593 Can PK/PD Modeling and Simulation Bring a Gene Therapy (for β-thalassemia) to Patients-in-Need Faster?

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Introduction: β-thalassemia is a rare hereditary genetic blood disease caused by mutations in the β-globin gene, resulting in reduced or absent production of functional hemoglobin. The standard care for patients with TDT requires regular (lifelong) transfusions of packed red blood cells. Previously, the only potential curative option in patients with transfusion dependent β-thalassemia (TDT) was allogenic hematopoietic stem cell (HSC) transplantation. Recently the FDA approved an autologous transplant/ex-vivo gene therapy beti-cel (betibeglogene autotemcel; commercial brand name: Zynteglo®), beti-cel is a gene therapy designed to add functional copies of a modified β-globin gene into TDT patients own HSC's. beti-cel consists of autologous CD34+ cells containing HSCs transduced with lentiviral vector encoding the $\beta^{A\text{-}T87Q}$-globin gene and produces hemoglobin containing $\beta^{A\text{-}T87Q}$-globin (HbA^{T87Q}). Peripheral blood vector copy number (PB VCN, expressed as average number of vector copies per diploid genome; c/dg) is a measure of drug product (DP) vector copies that is used to monitor transgene integration. Over the course of clinical development, the manufacturing process was optimized from DP process 1 to DP process 2 (the commercial DP process), specifically the transduction step to increase the percentage of vector-positive transduced cells (%DP LVV+ cells) and the total VCN. **Aims:** To develop a pharmacodynamic (PD) model to describe the PD profile of PB VCN and HbA^{T87Q} post beti-cel infusion. To identify and quantify both patient and DP specific factors that impacted PB VCN and HbA^{T87Q} time course as well as to determine the time required for both biomarkers to approach steady-state and thereby understand the dose-exposure-response relationship for beti-cel. **Methods:** As of 09Mar2021, data were available from 59 patients (n=39 non-β^0/β^0 genotype, n=20 β^0/β^0 genotype) enrolled in three clinical studies (HGB-204, HGB-207, HGB-212) and the long-term follow-up study (LTF-303). Patients were aged 4 - 35 years, weighed 16.3 - 95.4 kg, with 26 males and 33 females. A total of 18 patients received DP Process 1 and 41 patients received DP Process 2. A total of 566 PB VCN and

HbA^{T87Q} 560 observations were analyzed using a non-linear mixed effects modeling approach in NONMEM (Version 7.4, ICON, Hanover, MD). **Results:** DP manufacturing process and %DP LVV+ cells were identified as statistically significant covariates effecting the steady-state values and the time to approach steady-state for PB VCN and subsequently HBA^{T87Q}. Patients that received DP Process 1 compared to patients that received DP Process 2 were predicted to have a lower steady-state PB VCN (0.336 c/dg vs 1.5 c/dg) and HbA^{T87Q} (5.66 g/dL vs 8.85 g/dL) and a longer time to achieve 90% steady-state PB VCN (1.66 months vs 0.393 months) and HbA^{T87Q} (6.44 months vs 3.91 months). Age, weight, sex, race, or genotype did not impact PB VCN or HBA^{T87Q} time-course profiles. **Conclusions:** A one-time dose of beti-cel manufactured by DP Process 2, the commercial process, resulted in rapid increases in both PB VCN and HbA^{T87Q} (> 90% of steady-state values by 6 months) with durable responses observed out to the end of the follow up (up to 72 months). The non-linear mixed effects models developed were used to support the Biologics License Application (BLA) submission and may have facilitated the therapy being available to patients sooner.

1594 Designing Brain and Peripheral Tissue Regulatory Cassettes for rAAV-Based Canavan Disease Gene Therapy

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Canavan disease is a devastating autosomal recessive leukodystrophy that is caused by loss of function mutations in the aspartoacylase (*ASPA*) gene. The disease is usually lethal in the first few decades of life and has no approved treatments. Our lab has developed a recombinant adeno-associated virus (rAAV) vector-based gene therapy that shows efficacy in mice and is currently undergoing clinical trials. Like most approved rAAV-based gene therapies, the therapeutic *hAspa* transgene is driven by the ubiquitously active *CMV* enhancer/chicken β-actin (*CB*) promoter. The ideal gene therapy achieves transgene expression at therapeutic and/or physiological levels in the intended tissues, and negligible expression elsewhere. For rAAV-based gene therapies, transduced tissues are predominately defined by the tropism of the capsid and the activity of the promoter. Capsid engineering alone may not produce vectors that can confer the appropriate amounts of expressed transgenes across diverse tissues. Optimized regulatory cassettes must therefore be developed to direct transgene expression within the bounds of a capsid's tropism. We have identified a 3.4-kb sequence upstream of the mouse *Aspa* gene that we hypothesized to harbor distinct central nervous system (CNS) and peripheral tissue (PT) enhancers as demarcated by tissue-dependent epigenetic modifications. We predicted that these regions may be utilized in vector regulatory cassettes to enable tissue-specific transgene expression that mimics endogenous *Aspa* expression. We designed a series of luciferase reporter constructs containing the full *mAspa* promoter or combinations of the CNS and PT enhancers with the proximal promoter. Constructs were then transfected into HEK293 (embryonic kidney cells), N2A (neuroblastomas), or MO3.13 (hybrid oligodendrocytes) cultures. In MO3.13 cells, the *mAspa* promoter and its derivatives were found to be ten-fold more active than in HEK293s, and three-fold more active than in N2As. Importantly, the relative promoter activities observed in these *in vitro* studies trended towards expression expected of the endogenous *Aspa* gene. We next aimed to examine the *mAspa* promoter *in vivo*. The *mAspa*-derived promoters were cloned into *Egfp*reporter vector constructs and packaged into AAV serotype 9 (AAV9) capsids. We found that neonatal mice injected with vectors carrying the full 3.4-kb *mAspa* promoter (rAAV9-*mAspaP-Egfp*) by facial vein administration conferred nearly equivalent levels of reporter expression in the CNS and liver one month post-injection as mice injected with rAAV9-*CB-Egfp*. However, expression in skeletal and cardiac muscle was absent in mice injected with rAAV9-*mAspaP-Egfp* vectors, while animals treated with rAAV9-*CB-Egfp* vectors exhibited strong expression in these tissues. Surprisingly, the *mAspaP* vectors drove high levels of transduction in brown adipose tissue, while rAAV9-*CB-Egfp* conferred negligible transgene expression. Reporter gene expression in the hippocampus was observed to be most intense in the stratum lacunosum moleculare, while expression was completely absent in the granule cell layer of the dentate gyrus. In contrast, the rAAV9-*CB-Egfp* vector showed abundant expression in the granule cell layer of the dentate gyrus. Our ongoing studies aim to further characterize the *mAspa* promoter and its potential to drive transcription in a diversity tissues, specifically those impacted by Canavan disease.

1595 Systemic Administration and Transient Immunosuppression Eliminate Anti-Drug Antibody Responses Following Vectored Immunoprophylaxis in Mice

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The delivery of broadly neutralizing antibody (bNAb) transgenes using adeno-associated virus (AAV) is a promising strategy for preventing and treating infectious diseases. We have previously shown that AAV-mediated bNAb delivery can protect humanized mice from repetitive vaginal challenges with HIV. In addition, the feasibility of vectored immunoprophylaxis (VIP) as an approach towards sustained HIV suppression has been demonstrated in both humanized mice and non-human primates. Two separate clinical trials employing intramuscular administration of AAV-bNAbs have established the safety and tolerability of VIP. A more recent Phase I study (VRC603) has demonstrated that administration of AAV8-VRC07 results in long-term systemic expression of VRC07 in humans for up to three years. Despite these positive outcomes, these human studies have also identified infrequent development of anti-drug antibodies (ADA) against bNAb as a potential limitation on VIP efficacy. To understand the immune pathways leading to the induction of ADA and investigate new approaches to reduce ADA, we first assessed if the immunocompetent C57BL/6 mouse model could recapitulate the ADA response observed in human studies. Mice given intramuscular injection of 1×10^{11} genome copies (GC) of either AAV1-PG9 or AAV8-VRC07 showed that AAV1-PG9 elicited higher ADA response than AAV8-VRC07, analogous to the results of recent human studies. Similarly, the expression of bNAb was significantly lower in mice that

received AAV1-PG9 as compared to mice given AAV8-VRC07, which maintained high levels of expression for at least 12 weeks. To identify the immune cells responsible for inducing ADA and determine their impact on bNAB expression, we repeated these studies in various immune cell knock-out mouse models. We found that mice lacking B cells or CD4 T cells had significantly reduced ADA and dramatically higher levels of bNAb expression than immunocompetent animals. To evaluate the impact of the route of administration on ADA and bNAb expression, we compared intravenous (IV) and intramuscular (IM) routes of vector administration and found that IV administration elicited significantly lower levels of ADA and higher expression when compared to IM administration. Finally, we assessed the effect of two immune-suppressing drugs, dexamethasone, and cyclosporine, in inhibiting the ADA response and bNAb expression following VIP and found that dexamethasone, but not cyclosporine, inhibited ADA responses and increased antibody expression in the C57BL/6 mouse model. Overall, our finding demonstrates the utility of the C57BL/6 mouse as a pre-clinical animal model capable of recapitulating key aspects of the ADA responses observed in humans. Importantly, the route of AAV administration had a significant influence on ADA response and bNAb expression. Our results also highlight the feasibility of reducing ADA through transient immunosuppression using dexamethasone.

1596 Co-Opting the *SOCS3* Promoter for NK Cell Therapy Using Non-Viral Precision Genome Engineering

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Natural Killer (NK) cells are a type of innate immune cell able to clear viral infection and malignancy. NK cells represent an allogenic source of immune cells for cancer therapy that perform MHC-independent cell killing, highlighting that NK cells hold a promising future for off-the-shelf cell therapy. Genetic engineering offers further opportunities to enhance the potency of NK cells for immunotherapy. To improve persistence *in vivo*, armoring NK cells with continuous cytokine signaling is essential. Furthermore, chimeric antigen receptors (CAR) can boost the potency of NK cells by providing antigen-dependent activation. Armored CAR-NK cells have been proven effective for liquid tumor treatment and are being tested for effectiveness against solid tumors in several trials. However, uncontrolled constitutive CAR/cytokine expression will lead to off-target toxicity and cytokine storm. In light of this, an inducible promoter is a powerful approach for controlling CAR/cytokine expression in a cytokine-induced manner. Traditional inducible promoter systems rely on antibiotics or small molecule administration, such as doxycycline-inducible and cumate-inducible promoters. Even though these systems have been proven effective *in vitro* and in xenograft mouse models, the usage of xeno-derived genetic components, short half-life caused by liver detoxification, and development into antibiotic resistance remain unsolved. Moreover, vector design of traditional inducible promoter systems often requires the expression of additional transcription factors accompanied by minimal promoter elements in the construct and is restricted to viral delivery methods. To circumvent limitations in current inducible promoter systems, we repurpose the *SOCS3* locus as a cytokine-inducible promoter system by genetic knock-in using non-viral CRISPR-Cas9 genome editing. Synthetic genes driven by *SOCS3* promoter can be induced by cytokines, such as IL-2 and IL-21, which FDA has approved for cancer treatments. Compared to traditional inducible promoter systems, *SOCS3* promoter is purely dependent on the trigger of human-derived cytokines and independent from xeno-derived components. As a proof-of-concept, we knock-in a fluorescent protein into the *SOCS3* locus and systematically profile the regulation of *SOCS3* promoter by different cytokines. Under the optimized cytokine induction, synthetic genes inserted in the *SOCS3* locus can be stably expressed, which also sheds light on autonomous genetic circuit design. Leveraging the pre-existing cytokine-inducible regulation, we aim to engineer state-of-art inducible CAR-NK cells by targeting CAR and other therapeutics genes into the *SOCS3* locus.

1597 Development of a Novel Cell Culture Medium for AAV Production with Multiple HEK293 Lineages and Process Optimization

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Adeno-associated virus (AAV) has become a prominent vector for gene delivery to treat life-threatening genetic diseases. One of the major limitations for product development and treatment availability is the need for high levels of infectious AAV particles, therefore, media and process optimization is vital to achieving high AAV titers. Specific raw materials found in many cell culture media formulations, such as iron citrate, are known to interfere with PEI transfections and must be removed/reduced to enable efficient transfection. Therefore, media development work for HEK293 production of AAV via PEI transfections should target maximizing viable cell density (VCD) and AAV titer, while also enabling PEI transfection. To maximize AAV titers across multiple HEK293 cell lines, media development for AAV production was performed with three HEK293 cell lines with different growth rates. Following media development, transfections were optimized with Design of Experiment (DoE) studies. Finally, bioreactor parameters for growth, PEI transfection, and AAV production were evaluated separately in an Ambr® 15 bioreactor. With this approach, the highest VCD and AAV titers can be achieved without the bottleneck of a non-optimized step in the AAV production process.

1598 AAV-Mediated Gene Therapy Corrects the Severe Phenotype of Acid Ceramidase Deficient Mice

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Acid ceramidase deficiency is a group of autosomal recessive lysosomal storage disorder caused by mutations in the *ASAH1* gene that codes

for acid ceramidase (ACDase). This enzyme cleaves ceramide into sphingosine and fatty acid. These ASAH1-related disorders present with a spectrum of clinical manifestations during early (Farber disease, FD) or late infancy/ teenage (spinal muscular atrophy with progressive myoclonic epilepsy, SMA-PME). To date, there is no curative treatment for patients and therefore a clear unmet medical need. We recently established proof-of-concept studies that intravenous administration of a recombinant AAV9 vector expressing human ACDase in *Asah1*$^{P361R/P361R}$ mice at pre- and post-symptomatic stages of the disease is able to prolong the lifespan and correct the phenotype. Here, we performed a dose escalation study in *Asah1*$^{P361R/P361R}$ mice to determine the minimum effective dose (MED) of this vector. Three doses of AAV9-*ASAH1* were administrated intravenously in mutant mice at a late stage of the disease and the effect was analyzed at the clinical, molecular and histological level for a 6-month period. We found that the low dose was suboptimal, whereas most pathological parameters were corrected at mid dose. However, detailed histological analysis of treated mice 6 months post-injection revealed that administration of the vector at the high dose was able to avoid the presence of inflammatory infiltrates in some tissues, including the central nervous system. These results pave the way for preclinical development and clinical translation of this treatment in patients with Farber and SMA-PME diseases.

1599 Engineered Retrogradely Transported, Barcoded AAVs Elucidate Molecular Pathways Involved in the Pathogenesis in Parkinson's Disease (PD)

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Here we describe a novel strategy based on AAV engineering and transgenic animals. The MNM008 AAV capsid, capable of retrograde transport in DA neurons, is injected in the striatum of TH-Cre rats and DAT-Cre mice. The vector carries uniquely barcoded aSyn or non-pathogenic tagBFP.Injection into the striatum with Cre-recombinase restriction of the expression to DA neurons of the SN, we avoid possible injection-related inflammation and cell loss. The nuclei from the micro dissected SN area are enriched for DA neurons using FANS. This is achieved thanks to the viral vector prep containing a Cre-inducible histone-linked GFP (H2B-GFP), which is only expressed in the population of interest. Additionally, we used a recently adopted marker (Nurr1) in FANS to study the dopaminergic neurons in WT animals. We tested two emerging and one mature single-cell sequencing approaches. The first one is a single-cell combinatorial fluidic indexing protocol (Scifi) which recently showed to increase the 10X Genomics cells load capacity to 100 folds. The second one is using Parse Bioscience technology, which appends barcodes to each transcript by progressing cells through split-pool combinatorial barcoding steps. This allows to sequence up to 1 M using any standard biology lab equipment. The third approach is using the gold standard 10X Genomics that has been widely adopted in single-cell studies. The molecular barcoding will allow us to accurately measure the aSyn expression level in each DA neuron arranging them into disease stages in what we call pseudo-time. Moreover, this approach will identify causative cascades of changes at the transcriptional level.

1600 Evaluation of an Amphiphilic Graft Copolymer as a Non-Viral Vector for CRISPR/nCas9 on Lysosomal Storage Diseases

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CRISPR/Cas9 has become the current standard for genome editing in several fields. In gene therapy, as observed with the classical approaches, one of the main bottlenecks is the delivery of the genetic material. Although viral vectors show high delivery efficacy, they have been associated with significant side effects, difficulties during scaling-up, and high costs. In this sense, it is important to identify novel vectors that can facilitate the implementation of a greater number of gene therapies, among which nanotechnology-based non-viral vectors represent an important alternative. Previously, we demonstrated that a magneto-liposome vector may allow the delivery of a CRISPR/nickaseCas9 (nCas9) system for the treatment of different lysosomal storage diseases. However, a short shelf-life time of this vector may preclude its use in a clinical scenario. As an alternative in this study we evaluated a novel non-viral vector based on methoxy poly(ethylene glycol) and a hydrophobic block of poly(ε-caprolactone-co-propargyl carbonate) grafted with a predetermined number of poly(2-(dimethylamino) ethyl methacrylate) segments (hereinafter PP6D5). This vector was evaluated for two lysosomal storage diseases (MPS IIIB and Tay-Sachs disease). PP6D5 showed an IC_{50} between 0.99, 0.96, 0.07, 0.013, and 0.007 mM in HEK293 cells, human wild-type fibroblasts, 3T3 mice fibroblasts, U87MG astrocytoma, and SHSY5Y neuroblastoma, showing that central nervous system (CNS) cells are more sensitive to this polymer that fibroblasts. Transfection efficiency evaluation showed that PP6D5 has a similar o even higher transfection rate within the same cells (between 9 and 32%) compared to a lipofectamine-mediated transfection (between 2.6 and 25). For MPS IIIB, gene editing showed higher NAGLU activity levels in HEK293 cells (4 U/mg) and MPS IIIB fibroblasts (4.4 U/mg) compared to lipofectamine (2.3 and 3.6 U/mg, respectively), while in CNS cells increase in enzyme activity was only observed in astrocytoma cells. In the case of Tay-Sachs disease the effects on HexA activity varied depending of the evaluated cell. For instance, in 3T3 fibroblasts higher HexA activity levels (176 U/mg) were observed compared to lipofectamine transfection (132 U/mg), while in astrocytoma cells higher enzyme activity (965 U/mg) were observed with lipofectamine compared to PP6D5 (773 U/mg), and in neuroblastoma none of the strategies led to an expression of the protein. Overall the results showed that this polymer may represent a novel tool for the design of gene editing strategies based on non-viral vectors.

1601 Path to Clinic: Preclinical Pharmacokinetics, Biodistribution and Immunogenicity Strategies to Support First in Man Studies with VSV GP Oncolytic Virus

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The unique properties of oncolytic viruses (OVs) coupled with a dearth of regulatory guidance present challenges for their translation in industry. In particular, their replicative ability distinguishes them from many other therapeutic modalities, and is an important consideration when assessing pharmacokinetics (PK) and biodistribution (BD) in animal models. As their replication may be dependent on host disease state (e.g., tumor burden) and immune status (e.g., pre-existing immunity), tox/biodistribution studies are commonly performed in tumor-bearing animals. Here, we present strategies and approaches employed to bring Vesicular Stomatitis Virus pseudotyped with LCMV glycoprotein (VSV-GP) through IND-enabling tox/biodistribution studies to enable first-in-man trials. Preclinical IND-enabling studies were designed to capture peak periods of viral replication, shedding, and vector persistence, after both intravenous and intratumoral administration in mice. In addition, mechanistic studies were performed to quantify viral PK and replication using a UV-inactivated tool virus, as well as to assess the time course kinetics of immune responses after repeated immunization. For analysis of these studies, a diverse toolset was developed: RT-qPCR, infectivity assays, strand-specific *in situ* hybridization, viral inactivation, and anti-drug antibody (ADA) and neutralization assays. Overall, VSV-GP was well tolerated in healthy and tumor-bearing mice. The predominant distribution sites after intravenous administration were the spleen and liver. As expected, replication was highest in the tumor with minimal/abortive replication and a lack of secondary viremia observed in other tissues and blood. Novel molecular tools and *in vivo* approaches were developed to address the unique PK/immunogenicity considerations of a replicating oncolytic virus. Ultimately, these strategies resulted in successful translation of the VSV-GP platform to the clinic.

1602 Development of Novel Lipoplex Formulation Methodologies to Improve Large Scale Transient Transfection for Lentiviral Vector Manufacture

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Large-scale transient transfection has advanced significantly over the last 20 years, allowing the effective production of a diverse range of biopharmaceutical products, including viral vectors. New developments and improved transfection technologies are required to ensure that transient gene expression-based bioprocesses can meet the growing demand for viral vectors whilst continuing to consistently manufacture commercial grade viral vectors within strict tolerances for potency, purity, and safety, whilst ensuring high levels of regulatory compliance. When undertaking large-scale transient transfection processes there are challenges related to transfection reagent stability and lipoplex preparation times. We demonstrate the growth of cationic lipid-based liposomes, an essential step in many cationic lipid-based transfection processes, can be controlled through adoption of low pH (pH 6.40 - pH 6.75) and in low salt concentration (0.2X PBS), facilitating improved control over the nanoparticle growth kinetics and enhanced particle stability. Such complexes retain the ability to facilitate efficient transfections for prolonged periods compared to standard preparation methodologies. Liposomes prepared via conventional methods in cell culture medium maintained at a pH of 7.40 achieved particle sizes of 835 nm and 1311 nm after a 5-minute and 15-minute incubation period, respectively. The longer incubation corresponded to a significant reduction in transfection efficiency, from 54.5% to 37.4% ($p<0.0001$), and a 52% reduction in functional lentiviral vector titre ($p<0.0001$). Comparatively, liposomes prepared in culture medium maintained at pH 6.65 achieved particle sizes of 713 nm and 874 nm after prolonged 30-minute and 60-minute incubation periods, respectively, corresponding to equivalent transfection efficiencies of 62.5% and 60.2% ($p=0.9961$) and equivalent lentiviral vector titres ($p=0.6105$). Similar results were obtained when preparing liposomes in low salt concentration formulations. These findings have significant industrial applications for the large-scale manufacture of lentiviral vectors for two principal reasons. First, the alternative preparation strategy enables longer liposome incubation times to be used, facilitating effective control in a GMP setting. Second, the significant improvement in particle stability facilitates the setting of wider process operating ranges, which will significantly improve process robustness and maximise batch-to-batch control and product consistency.

1603 Preliminary Results from a Prospective, Multicentric, Follow Up Standardized Cohort to Assess Natural History of Duchenne Muscular Dystrophy

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Introduction Duchenne muscular dystrophy (DMD) is a rare monogenic and progressive neuromuscular disease characterized by functional decline from age 6, loss of ambulation between 8- 15 years of age, and early mortality. Multiple therapies aimed at delaying or stabilizing disease progression are in development. However, the heterogenous clinical course of the disease makes it crucial to understand baseline evolution of assessments to be measured in clinical trials. **Methods** We are conducting an international study in ambulant

DMD boys to assess longitudinally functional measurements prior to roll-over into a gene therapy clinical trial. Enrollment included patients aged from 5-9 years old who were able to cooperate with study assessments and achieved a North Star Ambulatory Assessment (NSAA) score ≥ 18 at inclusion. All patients were assessed using NSAA, timed function tests, MyoTools (upper limb muscles force tests), cardiac and respiratory function tests every 6 months. The analysis here includes 48 patients with at least 6 months follow up (mean= 13.5 months) from France and UK. Descriptive statistics and linear mixed effect model were used to describe the evolution of outcomes. Correlation analyses (Spearman) of these endpoints were performed. **Results** At inclusion, patient's mean age was 7.10 ± 1.37 years, 95.7% were receiving oral steroid treatment (mean duration:14 months), mean weight was 21.76 (±4.03) kg and mean height was116.43 ±9.34 cm. The mean total NSAA score was 25.52 ± 4.24, and mean time to Raise from Floor (TTRFF) was 4.43 ± 1.51s, with NSAA means being similar between patients < 7 and ≥7 years of age at screening. mean Forced Vital Capacity (FVC) and Forced Expiratory Volume in one second (FEV1) were 90.35±18.85 % predicted and 92.83±17.72 % predicted. Physical function tests scores were mostly stable or improving over time in younger patients, whereas they tend to decrease in older patients: e.g., NSAA slope of -0.07 (95% CI: [-1.79; 1.65] p=0.9341) in patients < 7yo and -3.19 (95% CI: [-5.03; -1.35] p=0.0011) in those ≥ 7yo at 12 months. The same trend was observed in pulmonary tests, suggesting early respiratory muscle decline at time of lower limb decline. NSAA, Time to 10 Meters Walk/Run Test (10MWT) and TTRFF correlated well between each other (Rho between 0.75 and 0.81). Upper limb force measurements (Myopinch and Myogrip) remained stable during follow-up and were not correlated with other functional tests, consistent with other reports of the trajectories of upper limb decline occurring later in disease progression. **Conclusions** This is a natural history study in DMD with a standardized follow-up and outcome measurements. The findings reinforce the need to include multi-measures for accurate outcome assessment in clinical trials with DMD, as different assessments decline at different rates and at different timepoints. It also supports to use NSAA as primary endpoint in this younger ambulatory population. These early results suggest that the monitoring of respiratory function may be useful to detect early divergence from expected trajectories for study-drug evaluation. More longitudinal data are being collected for a more robust and comprehensive analysis.

1604 Evaluation of Transcytosis Receptor Expression Between Human Brain Endothelial Cells *In Vitro* and *In Vivo* with Respect to Adeno-Associated Virus Capsid Selection

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The ability of adeno-associated virus (AAV) capsids to pass from the blood to the brain after intravenous infusion is crucial for being able to treat genetic diseases of the brain. Movement of AAV capsids across the blood-brain barrier (BBB) relies upon the process of transcytosis, in which the AAV capsid binds to a particular transcytosis receptor in the blood vessel lumen and transports through an endothelial cell into the parenchyma of the brain. Since naturally occurring serotypes of AAV have limited ability to undergo transcytosis, many efforts are ongoing to engineer capsids with increased transcytosis efficiency. One powerful engineering method is directed evolution of AAV capsids, in which a randomized library of genetically modified capsids is put through a selection process to identify new capsids with specific properties. The selection process utilized determines the properties of the resulting capsid(s), with notable cases where capsids selected to cross the BBB *in vivo* in mice have properties that do not translate to other species. Recently, our lab used a human transwell system, which allows for selection of capsids that can cross the human BBB, rather than a model organism. After isolating subsets of shuffled capsids that crossed the BBB and had a bias for transducing either astrocytes or neurons, an siRNA screen was performed to identify known human transcytosis receptors that might facilitate transport of these novel capsids. In the current study, we sought to better understand transcytosis receptor gene expression in hCMECs. To generate an overview of gene expression in the cells, mRNA-seq was performed on cultured wildtype hCMECs (n = 6). Surprisingly, for *MSR1*, the leading transcytosis receptor candidate from the siRNA screen, transcripts were not detected, at 0 transcripts per million (TPM). The next top five hits from the siRNA screen, *SCARB1*, *SCARA3*, *IGF1R*, *FCGRT*, and *LEPR*, were measured at 68, 90, 142, 18, and 80 TPM, respectively. These relatively low expression levels contrast with the mostly highly expressed putative transcytosis receptors we noted, which were *BSG*, *SLC7A5*, *PICALM*, *TFRC*, *IGF2R*, measured at 527, 354, 339, 263, and 231 TPM, respectively. We cross-referenced this mRNA-seq data to the most robust online human brain vascular atlas and found that *IGF1R*, *PICALM*, *BSG*, and *SLC7A5* had the highest expression from a set of putative transcytosis receptors in arterial, capillary, and venous cells, sharing some similarity with relative expression in hCMECs. In addition, the atlas revealed that the *MSR1* gene is only significantly expressed in peripheral macrophages *in vivo*, rather than vascular cells. In comparison to humans, the literature shows that *IGF1R* is expressed at the same relative level in mouse brain endothelial cells (BECs), however, *PICALM* is expressed at approximately twice the level in human BECs compared to mouse BECs. Overall, the data presented here show that a complete receptor knockout is important to validate artifacts that may arise from an siRNA receptor knockdown screen, generation of an mRNA-seq library of cells used for selection is an informative tool, and there are interesting parallels between human transcytosis receptor expression *in vitro* and *in vivo*. Future work will include creation of new knockout hCMECs to gauge interaction with AAV capsids and measurement of transcytosis receptor transcripts from the brains of non-human primates that will be used for preclinical testing.

1605 Production of High AAV Titers in Upstream Manufacturing Using Small Molecule Enhancers

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In recent years, there has been an exponential increase in the number of registered clinical trials examining the use of recombinant adeno-associated virus (AAV)-based gene therapies. Upstream production of AAV therapies is driven by transient transfection of suspension cells with multiple plasmid DNA constructs

encoding essential virus proteins. AAV therapies require high titers of viral genomes per dosage, prompting the need for more efficient methods of AAV manufacturing to reduce overall costs. Recently, we identified a library of small-molecule enhancers which can be optimized in conjunction with a variety of upstream AAV production parameters. These cutting-edge small molecules enhancers were tested with state-of-the-art transfection technologies developed within our group. DOE was performed to find the optimal conditions in which each small molecule would produce the highest AAV titers. These small molecules were not detrimental to transfection efficiency and each produced high-quality titers across a range of suspension HEK 293 subtypes, cell growth media, and cell densities as measured by viral genomes and the percentage of full capsids. To address scalability, we performed experiments in a stirred tank bioreactor (STR) up to 2L. Additionally, there was no difference in the effectiveness of these small molecules across a range of different plasmid DNA vectors encoding essential genes for recombinant AAV production. Multiple serotypes were assessed, including AAV2, AAV5, AAV8 and AAV9. Interestingly, certain small molecules were more effective at increasing AAV titers in some serotypes over others. Overall, our data demonstrate that due to the complex nature of AAV manufacture, it is advantageous to screen different small molecule enhancers during process development. Improvement of titers has broad implications for the economical manufacturing of high dose assets and mainstream utilization of AAV gene therapy.

1606 Detection of Gene Editing of the Dystonia (DYT1) Mutant Allele with Extracellular Vesicles

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DYT1 dystonia is a hereditary neurological movement disorder with a 3-bp hallmark deletion (c.907 909delGAG) in the *TOR1A* gene. This dominant-negative mutation is believed to compromise function of normal torsinA. TorsinA is located in the endoplasmic reticulum and has a role in protein processing, transport across the nuclear envelope and movement of nuclei. In this study, we aim to lower mutant torsinA levels and restore healthy torsinA activity in the basal ganglia of mouse brains. We used our previously established gene editing approach to disrupt the DYT1 allele with an engineered Cas9 variant from *Staphylococcus aureus* (SaCas9-KKH). As readout for effective disruption of mutant torsinA allele in DYT1 patient fibroblasts and human neuropreursor cells (hNPCs) derived from patient induced pluripotent stem cells, we analyzed the ratio of mutant and wild type torsinA mRNA in extracellular vesicles released from these cells. EVs were isolated from cell media and their RNA payload was assessed with a newly developed TaqMan-probe-based assay which distinguishes between the WT and mutant torsinA mRNAs. This readout was matched to the genotypic situation in the EV-donor cells. We are currently engrafting the brains of mice with patient-derived hNPCs prelabeled with a CRE reporter as a gene therapeutic screening method injecting AAV- CRE vector. EVs released from implanted cells into the bloodstream are isolated and evaluated using the TaqMan assay in addition to next-generation CRISPR sequencing as a readout for % of Cre-mediated editing. Overall, our technique provides a method to screen the efficacy of changing gene sequence in patient-derived cells implanted in the brain in response to gene therapy treatment, which is applicable to testing this genome-modifying therapy in preclinical DYT1 models.

1607 Preclinical Efficacy and Safety Study of AAVrh10-*GRN* Gene Therapy to Treat Frontotemporal Dementia Caused by Granulin Mutations

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Frontotemporal dementia (FTD) or frontotemporal lobar degeneration (FTLD) is one of the most common causes of early onset dementia. The disease is characterized by progressive degeneration of neurons in the brain's frontal and temporal lobes leading to changes in personality, behavior, and language. Up to 10% of FTD cases are caused by dominant loss-of-function mutations in the granulin (*GRN*) gene, resulting in > 50% reduction of progranulin levels. Currently, there are no disease-modifying therapies available for patients with FTD-*GRN*. In this study, we evaluated the efficacy of adeno-associated virus (AAV) gene therapy for the treatment of FTD-*GRN* by delivering AAVrh10-*GRN* (AGTC-601) to the CNS of adult homozygous Grn^{R493X} mouse model ($Grn^{R493X/R493X}$) via intracerebroventricular (ICV) injection. GLP Toxicity and biodistribution studies are in progress in adult Cynomolgus Monkeys following a single intracisterna magna (ICM) administration of AGTC-601 into the cerebrospinal fluid (CSF) at different doses. Grn^{R493X} treated mice did not show any behavioral abnormalities throughout the entire study. Human progranulin (hPGRN) expression levels were sustained in mice brain including Frontal Cortex, Hippocampus, and Thalamus for up to 6 months. The treatment rescued the lysosomal dysfunction in Grn^{R493X} mice by reduction of the accumulated lysosomal lipofuscin and the decrease in the elevated LGALS3 (Galectin-3) levels in mice brain. The ICM delivery of AGTC-601 in NHP was well tolerated with no associated adverse findings in-life at the tested doses and resulted in a sustained expression of hPGRN in the CSF for > 6 months. Interestingly, the use of a neuron-specific promoter restricted hPGRN expression to the CNS. Together, these studies support further development of AGTC-601 as a potentially one-time treatment for FTD-*GRN*.

1608 Functional Human Ovarian Granulosa-Like Cells Generation from Stem Cells via Network-Informed CRISPR Activation Screening

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Human granulosa cells (hGCs) play a key role in female reproduction by regulating human oogenesis and folliculogenesis in the ovary. The dysfunction of granulosa cells can lead to disorders of human ovarian function, such as polycystic ovary syndrome, premature ovarian failure, and granulosa cell tumors. However, the lack of reproducible cellular systems suitable to investigate the molecular mechanisms underlying ovarian development in

humans have hampered studies on granulosa cell differentiation. In this project, we create a novel system to differentiate human inducible pluripotent stem cells (hiPSCs) into hGC-like cells (hGCLCs) in vitro via activation of key transcription factors (TFs). We first employ a graph theory-based strategy, by constructing a gene regulatory network (GRN) representation of natural hGC differentiation and then employing centrality algorithms to prioritize the critical TFs that govern the hGC GRN. To experimentally validate TFs, we then leverage the CRISPR activation (CRISPRa) system for high-throughput screening. By employing fluorescent reporters of several key marker genes, such as FOXL2 and AMHR2, we validate master regulators of hGC fate specification, such as NR5A1, RUNX2, and GATA4, and further isolate guide RNAs activating novel regulatory TFs that enhance hGC differentiation. Furthermore, via lentiviral integration of identified TFs, we conduct functional and molecular phenotyping of hGCLCs and detect high levels of estrogen and progesterone, indicating that our derived hGCLCs retain characteristics of endogenous ovarian granulosa cells. Overall, we envision that our rapid 5-day hGCLC induction protocol will serve as a significant milestone in the effort to generate human ovarian follicles in vitro and establish a deeper understanding of the female reproductive system. Moreover, our system may also serve as a replenishable reservoir for healthy hGCs and enable potential cell therapeutic solutions for ovarian diseases.

1609 *In Vivo* Gene Editing of Murine Hematopoietic Stem Cells via AAV-Delivered CRISPR Guide RNAs

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Hematopoietic stem cells (HSCs) are the self-renewing, multipotent, and engraftable source of all cells in the blood system. Accordingly, hematopoietic stem cell transplantation (HSCT) has been utilized as a definitive cure for hematological disorders. Although HSCT has been improved upon for decades, associated complications still make this approach a last-resort option in many cases. Efficient *in vivo* gene editing of hematopoietic stem cells (HSCs) has the potential to eliminate the requirement for HSCT while still leveraging the potency of HSCs for blood cell repopulation in treating monogenic blood disorders. Towards this aim, we used adeno-associated virus (AAV) to deliver CRISPR guide RNAs (gRNAs) to gene edit HSCs *in vivo* in an Ai9;Cas9-eGFP reporter transgenic model. This model carries a modified ROSA26 locus, in which one allele encodes a Cas9-activatable Lox-STOP-Lox (LSL)-TdTomato reporter cassette and the other a constitutive SpCas9-2A-GFP. We tested whether systemic administration to these mice of AAVs carrying SpCas9-compatible gRNAs designed to induce cuts upstream and downstream of the STOP cassette would result in TdTomato expression in HSCs. Administration of AAVs carrying one copy of each guide ("1x gRNAs") in adults yielded rare, but readily-detectable, gene edited HSCs. We then tested whether modifications to our vector system might improve editing rates by utilizing a) self-complementary AAVs (scAAVs) and b) additional guide copies for the upstream and downstream guides ("2x gRNAs"). scAAV-1xgRNAs improved editing rates by ~6-fold compared to single-stranded AAV-1xgRNAs. Inclusion of an additional copy of each guide also improved editing rates in immunophenotypic HSCs by ~25%. With these modifications, we reached ~5% HSC gene editing in adult animals injected with scAAV-2xgRNAs. Furthermore, we found ~20% editing in HSCs when neonatal animals were injected with scAAV-2xgRNAs. To apply our approach to a disease-related gene, we generated sickle-cell disease (SCD) mouse models ("Townes" model) expressing SpCas9-eGFP from the ROSA26 locus, referred here as SCD;Cas9-eGFP. These animals have mouse HBA and HBB replaced with human HBA, HBB or HBS (healthy or sickle HBB), and HBG. Mice homozygous for HBS show SCD phenotypes similar to SCD patients. Studies are currently ongoing to determine whether we can apply our *in vivo* gene editing system to disrupt the binding site for BCL11a (a silencer of fetal hemoglobin) in the human HBG promoter of these mice, thereby elevating fetal hemoglobin production, which has been put forward as a therapeutic strategy to combat SCD. In conclusion, we introduce a system for *in vivo* gene modification of HSCs in mice and define several necessary components required for robust editing.

1610 Gold Nanoparticle-Mediated Gene Editing for Long Transgene Delivery *In Vivo*

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Gene editing using CRISPR has gained traction for its potential to treat various diseases. However, current gene editing therapies are hampered by delivery. While gene-edited cell products engineered ex vivo are largely effective, they are time and resource intensive to produce. Development of an effective in vivo therapy could overcome these limitations. Our lab developed a gold-based nanoparticle to simultaneously deliver CRISPR RNP and ~100bp single-stranded (ss)DNA templates (CRISPR-AuNP). Thiol-modified guide (sg)RNA binds a Cas12a nuclease to the AuNP core. This is coated in a cationic polymer to mediate endosomal escape. This polymer is coated in DNA template. Recent studies show this nanoparticle can carry templates as long as ~2.5kb. Here we tested CRISPR-AuNP for in vivo gene editing of the human immunoglobulin heavy chain (*IGH*) locus to introduce an engineered transgene. To evaluate the efficacy, we compared two formulations, A and B, based on characterization of our published CRISPR-AuNP formulation [2023-A-1421-ASGCT]. Formulation A followed published synthesis procedures. AuNP were incubated with modified sgRNA prior to conjugation with Cas12a. These particles were then coated in polyethylenimine (PEI) and incubated with double stranded (ds)DNA templates encoding a CMV-GFP reporter construct with homology arms to the human *IGH* locus. Formulation B was made by incubating pre-conjugated Cas12a RNP with AuNP, and then coating in PEGylated PEI prior to dsDNA addition to further stabilize the particle at physiological conditions. Both formulations were stable and monodisperse (A/B: hydrodynamic diameter =

65.9nm/64.8nm, polydispersity index = 0.19/0.18) in solution as measured by dynamic light scattering (**Figure 1B**). These CRISPR-AuNP were administered to humanized mice (**Figure 1A**) to evaluate delivery in vivo to hematopoietic cells. NOD/SCID/gamma null (NSG) mice were engrafted with human CD34+ cord-blood derived stem cells via intrahepatic injection at 1-2 days of age following sublethal total body irradiation. At 8 weeks, peripheral blood was evaluated for human cell engraftment and lineage development. Mice received three total CRISPR-AuNP injections bi-weekly beginning at week 9, each containing 1.5mg/kg of treatment A or B into the femur, the most accessible repository of human CD34+ cells in humanized mice. Injections alternated weekly with retroorbital bleeds. Following each injection we evaluated peripheral hCD45+hCD20+ B cells, the therapeutically relevant population for *IGH* editing. GFP expression was observed in 0.03% of B cells in group B mice (**Figure 1C**), increasing 0.12% and 0.23% respectively over the next month. Sequencing of the *IGH* locus in whole peripheral blood revealed indels at the target cut site with 2.4% overall gene editing for A and 5.2% for B (**Figure 1D**). Off target tissue analysis is ongoing. The successful expression of a long transgene in B cells in vivo suggests that CRISPR-AuNP are a viable method for in vivo transgene insertion at the *IGH* locus.

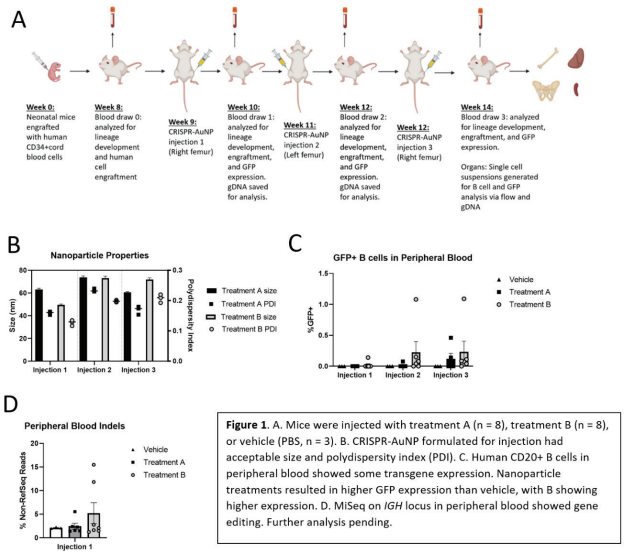

Figure 1. A. Mice were injected with treatment A (n = 8), treatment B (n = 8), or vehicle (PBS, n = 3). B. CRISPR-AuNP formulated for injection had acceptable size and polydispersity index (PDI). C. Human CD20+ B cells in peripheral blood showed some transgene expression. Nanoparticle treatments resulted in higher GFP expression than vehicle, with B showing higher expression. D. MiSeq on *IGH* locus in peripheral blood showed gene editing. Further analysis pending.

1611 *In Vivo* Editing of the *HAO1* Gene with ABR-001, a Novel Type V CRISPR Cas, Leads to Significant Reduction in Toxic Oxalate in a Mouse Model of Primary Hyperoxaluria Type 1

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Primary Hyperoxaluria is an inherited rare disease that results from the abnormally high production of the insoluble metabolite oxalate. This results in calcium oxalate crystal formation and deposition in the kidney and other tissues, resulting in painful kidney stones, renal failure, and other severe consequences of systemic oxalate deposition, traditionally treated by combined liver/kidney transplants or more recently, by lifelong siRNA therapy. Primary Hyperoxaluria type 1 (PH1), results from mutations in the *AGXT* gene, which encodes hepatic peroxisomal enzyme alanine glyoxylate aminotransferase (AGT) and converts the metabolite glyoxylate to glycine and pyruvate in the liver. In the absence of AGT enzyme, glyoxylate is converted to oxalate by lactate dehydrogenase. Glyoxylate, is primarily formed by the conversion of dietary glycolate by the enzyme, glycolate oxidase (GO), encoded by the *HAO1* gene. One strategy to treat PH1 is to block the activity of GO, reducing toxic oxalate burden by preventing formation of its precursor, glyoxylate and increasing production of glycolate, which is soluble and readily excreted compared to oxalate. Genetic inactivation of the *HAO1* gene through CRISPR Cas editing is an attractive alternative for treatment of severe PH1. Early exons of either human or mouse HAO1 genes were targeted with ABR-001, an engineered variant of Cas12i2, yielding indels in cultured cells. To understand the *in vivo* pharmacology of *HAO1* editing, synthetic modified guide RNAs (gRNAs) were packaged with an mRNA encoding ABR-001 in an LNP. A dose response study was performed in wild-type mice at doses ranging from 0.5-2.0 mg/kg total RNA. Editing, as determined by NGS, reached a maximum at 1 mg/kg and was primarily seen in liver. Characteristic of type V enzymes, the edits were primarily small deletions, predicted to disrupt protein production. Concomitant with editing, was a dose responsive rise in plasma glycolate (>10-fold above baseline), demonstrating expected modification of the metabolic pathway. Further studies were performed in a mouse model of PH1. LNPs, described above, were administered to AGXT deficient mice at doses of 1.5 and 3.0 mg/kg and mice were followed for three weeks. The level of plasma glycolate increased 8-10-fold following treatment. Importantly, the level of urinary oxalate was reduced by about 40%, to a therapeutically relevant range within one week of treatment and remained reduced throughout the three-week study. Editing, as determined by NGS, was approximately 60% for both groups, with slightly higher indels for the 3.0 mg/kg group. Analysis of GO enzyme activity in harvested liver fractions showed significant knockdown in the 1.5 mg/kg group and complete ablation in the 3.0 mg/kg group. These results provide *in vivo* proof of pharmacology for a gene editing approach to PH1 using a novel type V CRISPR Cas system.

[1]McGaw C and Garrity AJ, et al. Engineered Cas12i2 is a versatile high-efficiency platform for therapeutic genome editing. Nat Comm. 2022;13(1):2833.

1612 AAV Vector SKG0301 Exhibits Therapeutic Potential in a Pompe Mouse Model

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Pompe disease (glycogen storage disease type II) is a severe disorder caused by loss-of-function mutations in the human *GAA* gene encoding acid α-glucosidase. Deficiency of this enzyme results in accumulation of glycogen in lysosomes, impairing muscular and cardiac function. Enzyme replacement therapy (ERT) is the standard of care for Pompe disease, but this approach has several limitations including a short half-life of the drug that requires life-long bi-weekly infusions and an anti-drug antibody (ADA) response that leads to reduced efficacy.

To address these challenges, we explored the strategy of gene therapy by constructing and producing an AAV9 vector expressing a codon-optimized human *GAA* transgene, which would express GAA in muscle and cardiac cells in a durable manner upon a single intravenous administration. Among all the constructs we had screened, SKG0301 showed superior GAA expression levels in the target tissues with little immunogenicity. Systemic delivery of SKG0301 in adult *Gaa*$^{-/-}$ mice, a model of Pompe disease, resulted in a dose-dependent increase in GAA activity, clearance of glycogen in the muscles and heart, rescue of motor function, and extension of survival. These effects were lasting and therapeutically meaningful. In addition, the vector was well-tolerated by the animals. In summary, gene therapy is a viable approach for the treatment of Pompe disease. Our data suggest that SKG0301 is a promising drug candidate with potential therapeutic benefit for patients with the disease.

1613 A Sickle HUDEP2 Cell Model with GFP/BFP Tagged Beta-Globin to Examine Its Regulation in Treating β-Hemoglobinopathies and Disorders of Erythropoiesis

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Sickle cell anemia (SCA), a β-hemoglobinopathy, is a severe inherited monogenic disorder. It is caused by a point mutation in the beta-globin gene (*HBB*), leading to the replacement of glutamic acid by valine at the sixth residue. The resultant protein is hemoglobin S (HbS). One approach in treating SCA using CRISPR/Cas9 based gene editing relies on correcting the pathogenic point mutation in *HBB* via the homology-directed repair (HDR) pathway by delivering Cas9/gRNA and a corrective donor template. We found that, although HDR rates are high (~40%), about 42% of CRISPR/Cas9 induced DSBs are repaired via non-homologous end joining (NHEJ), resulting in small insertions and deletions (indels) at the Cas9 cut site as well as a high frequency of large gene modifications including large deletions (up to 4350bp). Small frameshift indels can introduce a premature stop codon and disrupt the expression of the target gene. With small in-frame indels the gene can still have transcription, resulting in proteins that are functional, partially functional, or pathogenic. Large deletions can cause gene disruptions, raising a concern of *HBB* gene knockout and the risk for known pathogenic phenotypes such as β-thalassemia. These CRISPR/Cas9 induced on-target modifications result in diverse and heterogeneous genotypes which may in turn lead to deleterious phenotypes. Due to this heterogeneity and short life span of terminally differentiated HSPCs, it is difficult to link a specific genotype to an observed phenotype. We aim to develop a fluorescent cell model to monitor allele specific *HBB* expression in live cells. With this tool, we will be able to assess the downstream consequences of *HBB* gene modifications and evaluate gene editing targets for SCA. Sickle Human Umbilical Cord Derived Erythroid Progenitor cells (S-HUDEP2) are immortalized CD34^{+} hematopoietic stem and progenitor cells with homozygous sickle mutation that exclusively produce HbS upon erythroid differentiation. We established a clonal S-HUDEP2 fluorescent model by labelling each *HBB* allele with a fluorescent protein (GFP or BFP) via tagging at C-terminus of *HBB* with a P2A self-cleaving peptide (HBB$^{GFP/BFP}$). In this model, GFP/BFP expression is under direct control of the *HBB* promotor and bi-allelic *HBB* tagging was confirmed by sequencing. To validate this model, we delivered HiFi *Sp*Cas9/sgRNA as ribonucleoprotein (RNP) via electroporation using the two sgRNAs targeting the sickle mutation currently in pre-clinical and early phase clinical trials (R-66S and R-02 respectively). Efficient editing was confirmed via Inference of CRISPR Edits (ICE) analysis. We conducted *in vitro* expansion of edited clones. Using FACS, we observed distinct populations based on GFP/BFP fluorescent intensity (negative, low, medium, high) in edited clones compared to mock treated controls. We sorted 4 distinct populations based on fluorescent expression: (i) GFP^{high}/BFP^{high}, (ii) GFP^{high}/BFP^{Low}, (iii) GFP^{low}/BFP^{high}, (iv) GFP^{low}/BFP^{low}. From each population, genomic DNA was extracted for digital droplet PCR (ddPCR) probe-based copy number assay to identify large deletions (>37bp) and for ICE analysis to identify small indels. These analyses confirmed enrichment of large deletions and frameshift indels in the GFP^{low}/BFP^{low} sub-populations and each group exhibited a unique indel profile based on GFP and BFP expression. The gRNA-specific indel profiles demonstrated our cell model's ability to identify diverse *HBB* repair outcomes. In summary, we have established a S-HUDEP2 cell model allowing for monitoring allele specific *HBB* expression based on fluorescence and directly correlating genotype and phenotype. This cell model can be used as a tool to evaluate the efficiency and safety of gene editing based therapy for SCA.

1614 Electroporation-Mediated Gene Delivery of the Na^{+},K^{+}-ATPase β1 Subunit to the Esophagus Induces Tight Junction Expression and Prevents Dilated Intercellular Space (DIS) Formation in a Rabbit Model of GERD

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Gastroesophageal Reflux Disease (GERD) is characterized by the erosion of the esophageal mucosal lining caused by recurrent stomach and bile acid exposure. In reflux disease gastro-duodenal contents regurgitate into the distal esophagus due to an incompetent lower esophageal sphincter. The bathing of the lower esophagus with stomach acid and bile acids results in the formation of dilated intercellular space (DIS) which is a pathological feature of GERD. DIS and the loss of barrier function of the epithelium caused by cellular injury also results in the disruption of tight junctions. We have found a reduction in transepithelial resistance (TEER) in our immortalized primary esophageal keratinocyte stratified squamous cell culture system upon treatments with bile salts at low pH. We have also seen a marked reduction in Occludin staining, a tight junction protein, in esophageal tissue samples from patients with GERD as compared to normal control tissue. These findings highlight the disruption in epithelial tight junctions under reflux disease conditions. Our laboratory has previously shown that overexpression of the β1 subunit of the Na^{+},K^{+}-ATPase results in the increased expression of tight junction proteins and restores epithelial barrier function in lung tissue.

Therefore, we hypothesized that maintaining and reinforcing epithelial barrier function in the distal esophagus through the modulation of tight junction proteins via gene delivery of the Na^+,K^+-ATPase β1 will prevent DIS and GERD symptoms in vivo. We have developed an electroporation-mediated gene transfer procedure to deliver naked plasmid DNA to the distal region of the esophagus in healthy rats and rabbits. Our gene transfer technique yielded robust transgene expression in rats as early as 48 hours post transfection (CMV-Luciferase) which persisted as long as 6 and 12 weeks (UbC-Luciferase) post transfection. We next tested our gene delivery procedure in the context of an established rabbit GERD model. In this system, Norwegian white rabbits are given GERD and DIS via a total 360° cardiomyectomy procedure which strips the controlling musculature of the gastric sphincter resulting in incompetent sphincter control and the formation of DIS by 12 weeks post procedure. We have delivered the Na^+,K^+-ATPase β1 subunit or a matched GFP control plasmid via electroporation at the time of cardiomyectomy to the exposed distal esophagus. After the completed surgical procedure and electroporation, the rabbits recover and are housed for a duration of 12 weeks. A clear induction of DIS is present in animals that underwent cardiomyectomy and received the GFP vector control plasmid. By contrast, rabbits that underwent cardiomyectomy and received the Na^+,K^+-ATPase β1 showed marked levels of protection from DIS formation as determined by electron microscopy**.** Our findings show that gene transfer of the Na^+,K^+-ATPase β1 subunit upregulates tight junction abundance and activity to improve barrier function in the esophagus and provides a degree of protection from development of DIS, a hallmark for GERD.

1615 Efficacy and Safety Assessments of AAV-Mediated CYP4V2 Augmentation for the Treatment of Bietti's Crystalline Dystrophy

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Introduction: Bietti's crystalline corneoretinal dystrophy (BCD) is a rare-inherited disease characterized by the presence of multiple shimmering yellow-white deposits in the fundus of patients in association with atrophy of the retinal pigment epithelium (RPE) and chorioretinal atrophy. The onset of the disease usually occurs between the teenage years and the age of 30. As the disease progresses, most patients become blind in the age of 40 to 50. BCD is caused by mutations in the cytochrome P450 (CYP) family 4 subfamily V member 2 gene (*CYP4V2*) encoding a polyunsaturated fatty acid hydroxylase which is dominantly expressed in RPE cells in retina. Currently, there is no treatment available for BCD. **Methods/Results:** We have developed NGGT001, an rAAV2-based vector expressing codon optimized human *CYP4V2* for treating BCD. Using CRISPR-Cas9 gene editing, we generated two human cell models carrying *CYP4V2* pathogenic mutations. These *CYP4V2* mutant cell line models exhibited several typical cellular defects observed in lesion-affected cells from patients with BCD, such as deficits in cell proliferative activity, autophagy, and lipid metabolism. Expression of CYP4V2 mediated by NGGT001 effectively rescued phenotype defects caused by *CYP4V2* mutations in these two BCD-relevant cell models, as demonstrated by restored autophagy flux activity, reduced lipid accumulation and preserved cell viability under lipid challenge, providing the therapeutic concept of function restoration by *CYP4V2* gene augmentation. Furthermore, stable vector transduction and long-term expression of CYP4V2 protein were achieved in the ocular compartments by a single subretinal injection of NGGT001 in WT mouse and in a knock-out mouse model of *Cyp4v3*, the mouse ortholog of human *CYP4V2*. In adult Cynomolgus monkeys administrated with subretinal injection of a single dose of NGGT001, robust vector distribution and sustained *CYP4V2* mRNA expression were observed in retinal and choroid tissues. Sufficient and persistent levels of CPY4V2 expression in the animal eyes supported the long-term therapy efficacy of function augmentation. In Brown Norway rats and in Cynomolgus monkeys following a bilateral single subretinal injection, NGGT001 induced limited inflammation and minimal test article-related changes in ophthalmic examinations and pathology during the 13-week toxicology studies. Vector DNA and transgene mRNA were mainly detected in ocular tissues, a small amount in liver and spleen and not in the whole blood. Thus, subretinal administration of NGGT001 was generally well tolerated. **Conclusions:** Taken together, our efficacy and safety studies demonstrated the safety of NGGT001 and highlighted that NGGT001 achieved stable expression of active CYP4V2 protein and restored lipid metabolism in the retinal cells. These results support the clinical deployment of NGGT001 as a promising gene therapy treatment for BCD.

1616 ATLAS: A High-Throughput Gain and Loss-of-Function Screening Platform for Optimizing AAV Production

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Recombinant AAV is one of the most widely used vectors for in vivo gene delivery. There are currently over 900 ongoing pre-clinical and clinical programs using AAV which all require significant manufacturing capacity. Inefficiencies in current manufacturing techniques has ultimately resulted in extremely high cost of gene therapy drug products. This severely limits the widespread use of AAV-mediated gene therapies for common and rare indications. In this study we present ATLAS (Arrayed Targeted Library for AAV Screening) a modality-agnostic, high-throughput screening platform for target identification for improved rAAV production. To enable screening in a miniaturized format, we first developed a highly sensitive cell-based potency assay that detects the presence of potent AAV9 with vector genomes as low as 2.5E8 vg/mL. We confirmed that our cell-based potency assay shows a strong correlation between capsid ELISA and qPCR-based assay. We used our cell-based potency assay to conduct high-throughput screening of small molecules and transgene over-expression libraries, resulting in the identification of a novel combination of factors that boosted rAAV9 production by over 30-fold in our clonal suspension HEK 293 compared to HEK 293F cells. Current efforts are underway to scale-up these findings to a 50L bioreactor.

1617 Gene Therapy Restores the Transcriptional Program of Fanconi Anemia Hematopoietic Stem Cells

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Fanconi anemia (FA) is an inherited disorder predominantly associated with bone marrow failure (BMF) and cancer predisposition. We have recently shown that lentiviral-mediated gene therapy in non-conditioned patients can progressively improve peripheral blood cell counts or stabilize these counts when used in early stages of the BMF. Here we have investigated whether defects in the transcriptional program of hematopoietic stem and progenitor cells (HSPCs) from FA patients can be corrected by gene therapy, or on the contrary are irreversible due to the intrinsic characteristics of the disease or because of accumulated DNA damage. To investigate this question, BM samples from four FA-A patients included in the FANCOLEN 1 trial were obtained 2 to 4 years post-gene therapy. $CD34^+$ cells were then purified and analyzed by single-cell RNA seq. Data was preprocessed using Seurat v4, and a machine learning framework was conducted to identify the different HSPC populations. Corrected and uncorrected $CD34^+$ cell populations coexisting in these patients were then classified based on the expression of FANCA. scRNAseq analyses allowed us to identify the presence of $FANCA^+$ and $FANCA^-$ cells within the different HSPC populations present in the BM of gene therapy treated patients. The transcriptional analysis of $FANCA^+$ and $FANCA^-$ cells showed statistically significant changes between these populations which were shared in the different HSPCs and reproducible in the four FA gene therapy treated patients. Furthermore, changes in the transcriptional program between $FANCA^+$ and $FANCA^-$ HSPCs from these FA patients resembled the ones noted when HD HSPCs were compared with $FANCA^-$ HSPCs. Finally, a gene-set enrichment analysis revealed that the genetic correction of FA HSPCs normalized several relevant pathways in FA through the upregulation of genes associated with DNA damage detection and repair, cell cycle, DNA replication, and telomere maintenance. Our results show that lentiviral-mediated gene therapy reprograms the altered HSPC molecular circuits in FA patients. Furthermore, our study reveals that the gene-expression fingerprint of corrected FA HSPCs resembles the transcriptional program of healthy HSPCs. These observations demonstrate for the first time that defects in the transcriptional signature and molecular pathways of FA HSPCs can be restored by means of lentiviral-mediated gene therapy.

1618 Development of Miniaturized CRISPR Activation for a Single AAV Delivery Method for Muscular Dystrophy

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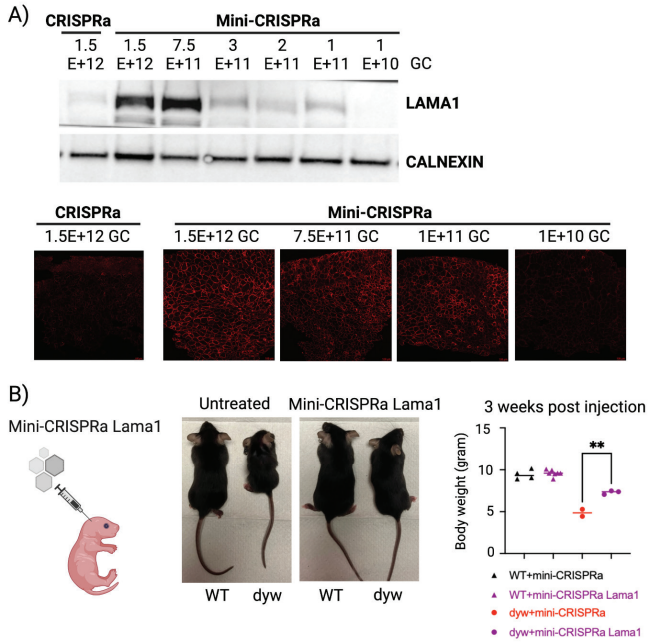
LAMA2-related dystrophies (LAMA2-RD) are a subtype of congenital onset muscular dystrophy caused by mutations in the *LAMA2* gene encoding laminin-α2, an extracellular protein essential for skeletal muscle and Schwann cell functions. Diseased correction is hampered by the mutation heterogeneity in patients, for which over 300 disease-associated mutations have been reported. In contrast, the upregulation of the compensatory gene *LAMA1* can serve as a mutation-independent approach applicable to all patients. We previously showed that upregulation of *Lama1* via AAV-mediated CRISPR-activation (CRISPRa) rescues disease phenotypes in mice. However, it required the use of dual-AAVs, which directly contributes to a high dose, toxicity, and cost. In this project, we aim to generate a mini-CRISPRa system suitable for a single AAV9 packaging to improve the clinical relevance of this technology. First, we swapped the commonly-used CMV promoter with a novel, 7.5x-shorter synthetic promoter termed 4xNRF1 to drive *Sa*dCas9 expression. Subsequently, we substituted the previously used 2xVP64 transcriptional activators with a miniaturized tripartite VP64, P65, and RTA activators (miniVPR), and coupled it with a single gRNA targeting the mouse *Lama1* promoter. Evaluation of the mini-CRISPRa system revealed robust *Lama1* upregulation in murine myoblasts. Furthermore, we observed successful *Lama1* upregulation in tibialis anterior muscles of LAMA2-RD mice (termed dyw) at the doses of 1.5E+12 AAV9 GC to 1E+11 GC; the latter dose was 15 times lower than previously reported (Figure A). Subsequently, we treated neonatal dyw mice with the mini-CRISPRa system via temporal vein injection. Compared to the untreated dyw, the treated dyw mice have reduced hindlimb paralysis, improved mobility, and body weight at 3 weeks post-treatment (Figure B). Current experiments involve quantitative analyses of neuromuscular functions, examination of survival, and immune responses upon treatments. Collectively, our data indicate that the mini-CRISPRa system is a more compact, yet more potent CRISPR activation system that shows significant promise in ameliorating the disease phenotypes in LAMA2-RD mice. The approach we developed opens up innovative therapeutic avenues based on the upregulation of compensatory genes that are potentially applicable to other diseases.



1619 Validating Reliability of Genome Editing Using Ion Channels as Drug Target

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Severe forms of inherited ocular channelopathy are caused by point mutations in *KCNJ13,* which affect the retinal pigment epithelial (RPE). We have previously demonstrated that both gene therapy and translational read-through result in functional recovery as tested using induced pluripotent stem cells iPSC-RPE from a patient line. AAV-gene therapy related immune responses, CRISPR/Cas9 gene editing associated off-targets, and indels pose some challenges in drug discovery and its clinical translation. We used Adenosine and Cytosine base editors (ABE8e, evoCDA-CBE & BE4max-CBE) along with a specific sgRNA as proof-of-concept correction of *KCNJ13* point mutations (c.158G>A [p.W53X] and c.431T> C [p.L144P]) using nanoparticle-mediated delivery to HEK293-stable cells, LCA16-patient's specific fibroblasts and iPSC-RPE. BE efficiency, protein localization and high throughput channel function were assessed in edited cells and compared with non-edited mutant and WT cells. Potential off-targets were screened to evaluate the accuracy and efficacy of BEs. CBE for L144P editing in stable cells resulted in high on-target editing (60-80%). However, bystander 'Cs' in the protospacer region led to either missense (~61%) or silent (~59%) variations in edited cells which affected the gene function. Very few reads had a WT gene sequence (~3%). ABE8e mRNA for W53X correction in stable cells showed 50% editing. Nanoparticle-mediated delivery of ABE8e in fibroblasts (47% editing) and iPSC-RPE (20%) established its use for *in vivo* BE delivery. On target indel mutagenesis (<3%) and screening of potential off-target sites (<1%) indicated high accuracy of the ABEs. Electrophysiology assays demonstrated in vivo functional rescue in a mouse model. **In conclusion,** sequence complexity of L144P locus poses some challenges to edit it with the current generation of CRISPR BEs. Using modified CBEs with tighter editing windows or using prime editing for the hard-to-rescue alleles would be beneficial. Functional studies to confirm the therapeutic potential of base editing is must. A caution is warranted to avoid detrimental outcomes of silent editing. W53X editing using ABE8e delivered via non-viral platform, silica nanoparticles showed specific editing without generating detrimental bystander substitutions, indels or off-target edits elsewhere in the genome. K^+ conductance in iPSC-RPE confirmed the functional rescue of the Kir7.1 channel following base editing. Altogether, these preclinical base editing studies for correcting mutations causing childhood blindness and other ocular genetic diseases hold promise for clinical translation.

1620 Optimizing Plasmid Complexation Parameters to Streamline AAV Vector Manufacturing

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Transient transfection of three plasmids (rep, cap, and helper) in HEK293 cells facilitated by a transfection reagent is the most widely used method for AAV and other viral vector production. It has, in fact, been shown effective for all AAV serotypes and is a regulatory-validated approach. Transfection reagents are necessary for transient transfection because naked DNA is not readily transported across cell membranes. The mixture of plasmid DNA with transfection reagent, or complexes, tend to be unstable and shear-sensitive, limiting the stirring speed for mixing, the rate at which they can be transferred from the mixing tank to the bioreactor, and the time they remain available for transfection into HEK293 cells. Optimization of the complexation processes is therefore essential to ensuring the efficiency and productivity of the overall transient transfection process leading to higher yield of viral vectors. The three main parameters that significantly impact the complexation process include: 1. The choice of complexation reagent, 2. ratio of transfection reagent to plasmid DNA, and 3. complexation time. Perturbations of these factors influence the size of the complexes generated, which has been linked to transfection efficiency across cellular membranes. Selection of the appropriate transfection reagent for use in recombinant viral vector production can directly impact the efficiency of the complexation step and ultimately the productivity during the transfection process. Reagents based on lipofectamine, polyethyleimine (PEI) and lipid-polymer complexes are now available. PEIPro® (Polyplus) has been widely used and is suitable for use with many virus types on both adherent and suspension cell culture platforms. FectorVIR® AAV (Polyplus) is a reagent designed specifically for suspension-based transient transfection of AAV vectors and is experiencing increasing use as the industry transitions to larger bioreactor processes. Both reagents are available in research and GMP grades, facilitating tech transfer from the lab to commercial

scale. FuGENE® HD and 4K (Promega Corporation) are additional multicomponent (cationic lipid polymer) transfection reagents for research-scale transient transfection in various mammalian cells (including both HEK293 and Chinese hamster ovary, or CHO) with GMP-readiness expected this year. To obtain a greater understanding of how the complexation process impacts down-stream yields of AAV, complexation reactions were performed over a range of time and reagent ratios using plasmids typically used in triple-transfection method for AAV generation. Down-stream purification using AKTA-based affinity and anion-exchange chromatography, coupled to analytical characterization of purified AAV vectors were performed. Herein we present how selection of these reagents directly impacts virus yield and recovery.

1621 Novel Buffer Formulations for Improved Recombinant Lentiviral Vector Stability and *In Vivo* Delivery to the Murine Lungs

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We have developed lentiviral vectors (LVs) based on recombinant Simian Immunodeficiency Virus pseudotyped with the Sendai Virus Fusion and Hemagglutinin-Neuraminidase glycoproteins (rSIV.F/HN). This vector platform has high *in vivo* gene transfer potency for the respiratory tract and directs sustained, high-level transgene expression after both single and repeated administration. We are developing rSIV.F/HN vectors for a range of acquired and inherited respiratory disorders. In our research-grade manufacturing process, rSIV.F/HN vectors are formulated via tangential-flow filtration (TFF) buffer exchange. In our cGMP-grade manufacturing process TFF is utilised in both the drug substance (DS) and drug product (DP) steps, both of which include a terminal hold at -80°C. Thus, the ideal vector formulation would provide protection from both TFF-induced shear and freeze/thaw steps. Our research-grade vectors have historically been formulated in TSSM buffer, composed of 20mM Tris pH7.3, 100mM NaCl, 10mg/mL sucrose, 10mg/mL mannitol, all reagents Generally Regarded As Safe (GRAS) by the FDA, and offers some freeze/thaw and shear force protection. In this study, we prepared rSIV.F/HN vectors in two new, proprietory formulation buffers (Buffer B and C, each generated with GRAS reagents) and compared their performance with vectors formulated in TSSM. We found that all three formulation buffers supported 3 freeze-thaw cycles with little or no loss of vector transduction (p>0.57). We also noted, however, a trend for a modest loss of vector activity as the number of freeze-thaw cycles was increased from 5 to 10 suggesting that additional formulation development may be necessary if further enhanced freeze/thaw stability is desirable. Dynamic light scattering studies suggested that TSSM and Buffer B imparted similar Zeta potential and particle size on our vectors, suggesting that colloidal stability would be similar. Encouragingly, we noted that extended holds (up to 7 days) at 4°C were possible in both TSSM and Buffer B without loss of vector transduction activity (p>0.99). This property may allow the removal of the -80°C hold between our DS and DP steps and simplify our manufacturing process. Interestingly, while TSSM and Buffer B supported brief (2h) excursions to 37°C with little or no loss of vector activity (p>0.33), vector formulated in Buffer C tended to show a modest loss of transduction activity. Nebulisation of the product is desirable for delivery to the lungs and, therefore, we evaluated vector transduction after passage through an Aerogen Solo vibrating mesh nebuliser. Encouragingly, vector formulated in both TSSM and Buffer B were largely unaffected by nebulisation (retaining 94.5% and 89.4% of their non-nebulised activity). To assess *in vivo* performance, we delivered matched quantities of rSIV.F/HN vectors expressing firefly luciferase formulated in TSSM and Buffer B to the lungs of BALB/c mice. Both formulations directed abundant lung transgene expression at indistinguishable levels (p=0.34). In summary, we have generated two new formulation buffers for LVs, one of which (Buffer B) supports multiple freeze-thaw events, extended holds at 4°C, and 37°C thermal challenge in a manner indistinguishable from the TSSM benchmark. Furthermore, vector formulated in Buffer B retained a high degree of transduction activity after nebulisation and directed abundant *in vivo* gene transfer. Thus, we find that Buffer B formulated rSIV.F/HN vectors warrant further investigation to establish their suitability for clinical development.

1622 Pentose Alcohol Metabolite Alters Gene Expression and Dystroglycan Glycosylation in a KRAS-Dependent Manner

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The protein α-Dystroglycan, and its post-translational O-glycosylation (matriglycan) enables its critical function in binding the muscle extracellular matrix. Without matriglycan, the functional capacity of dystroglycan as part of the dystrophin-associated protein complex is lost, and leads to pathologies known as the dystroglycanopathies. We have recently shown that the metabolite ribitol, a pentose alcohol, is able to upregulate functional glycosylation of α-dystroglycan in FKRP-mutant mouse models of Limb Girdle Muscular Dystrophy, and in breast cancer epithelial cells. We have also demonstrated that the effect of ribitol administration results in altered gene expression. Analyzing gene expression profiling, we have identified a number of candidate genes altered with administration of ribitol and found that the Kirsten Rat Sarcoma protooncogene (KRAS) may play a significant role in the glycosylation of α-dystroglycan. Using siRNA, we have found that suppression/knockdown of KRAS expression in the MCF-7 breast cancer cell line results in decreased expression of matriglycan-modified dystroglycan. Interestingly, this reduction in matriglycan-modified α-dystroglycan cannot be rescued by ribitol supplementation. The highly ordered process of O-mannoglycosylation of α-dystroglycan has been well characterized through the examination of the functional roles of more than 18 glycosyltransferase enzymes involved. However, it is not clear how expression of these genes is regulated in relation to cell proliferation, differentiation, and cellular functions. The ability of a single gene such as KRAS, however, to modulate detectable

matriglycan glycosylation of α-DG is unique and warrants further study for understanding of this process and for potential therapeutic applications for both the oncologic and dystrophy fields.

1623 Multiple *In Vitro* Differentiated Skeletal Muscle Models for Screening of Synthetic Muscle Promoters for Gene Therapy

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Gene therapy delivery to skeletal muscle for inherited musculoskeletal disorders has been challenging, in part due to the target tissue's large size, mass, and anatomical distribution, and the requirement for high systemic vector doses of adeno-associated virus (AAV) for therapeutic efficacy. High AAV doses have caused dose-limiting toxicity and immunogenic responses, as observed in ongoing clinical trials for inherited muscular dystrophies. Muscle tissue is also an effective target for the therapeutic production of secretory proteins, like enzymes, hormones, antibodies, and clotting factors, following local delivery of the viral vector containing the therapeutic transgene to the muscle. The potential of the muscle as a target for gene therapy highlights a demand for more potent, tissue-specific, and compact regulators of therapeutic gene expression, such as promoters. Stronger promoters can provide therapeutic potency at lower viral vector doses, while a reduction in size allows for more efficient packaging of larger transgenes or expression cassettes. To our knowledge, there is a lack of accessible data comparing transcriptomic profiles across multiple *in vitro* and *in vivo* skeletal muscle models pre- and post-differentiation, which can be used for designing and testing novel muscle-specific AAV therapies. Here, we present multiple *in vitro* differentiated myotube models of skeletal muscle for more efficient evaluation of engineered muscle promoters for gene therapy and their characterization by transcriptomics and immunofluorescence analyses. The differentiation-induced myotube models include C2C12 immortalized mouse myoblast cell lines, primary human myoblasts from the quadriceps, and human induced pluripotent stem cell-derived myoblasts. Transcriptomic changes were assessed before and after differentiation for each model and condition. After validating the presence of key myotube differentiation markers, a set of in-house proprietary muscle promoters were tested in these models and compared against known reference muscle promoters currently used in clinical trials of AAV-delivered treatment for muscular dystrophies. Top performing candidates from our rationally designed compact muscle-based promoters were evaluated in these *in vitro* skeletal muscle models, in addition to *in vivo* assessment in C57BL6 mice, with our results showing higher transgene expression compared to reference muscle promoters. In summary, we show promising reliable *in vitro* differentiated skeletal muscle models characterized via RNAseq and immunofluorescence as well as demonstrate the potential of our promoter engineering platform designed to target specific tissues of interest, like skeletal muscle

1624 A High-Fidelity RNA-Targeting Cas13 Restores Paternal Ube3a Expression and Improves Motor Functions in Angelman Syndrome Mice

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Background: Angelman Syndrome (AS) is a severe neurodevelopmental disorder, with an incidence of approximately 1/12000-1/20000 worldwide, which is characterized by seizures and severely impaired motor, cognitive, and language skills. AS is mainly attributable to loss of maternally inherited *ubiquitin protein ligase E3A* (*UBE3A*), which mediates the targeted degradation of several protein substrates. Only the maternally inherited UBE3A allele in neurons is active due to selective silencing of paternal *UBE3A* by its antisense transcript, *UBE3A-ATS*. **Methods:** We tested the knockdown efficiency of Ube3a-ATS in primary neurons isolated from AS mouse (Ube3am−/p+) using lentivirus packaged high-fidelity Cas13 (hfCas13x.1) and a crRNA (Fig. 1B). To evaluate the efficiency of Ube3a-ATS silencing in brain, AAV with hfCas13x.1/cr9 was injected into postnatal day 0-1 AS mice via intracerebroventricular (ICV) injection. The protein of reinstated UBE3A in dissected brain regions was evaluated by western blot (Fig. 1C, D). The body weight and behavioral tests were evaluated in AS mice with AAV-hfCas13x.1/cr9 (Fig. 1E-H). **Results:** We isolated and infected AS (*Ube3a*$^{m-/p+}$) primary cortical neurons with lentivirus expressing Cas13x.1-Flag and found that the UBE3A expression restored by hfCas13x.1/cr9 was enriched in the nucleus of AS mouse primary neurons (Fig. 1B). To evaluate the efficiency of Ube3a-ATS silencing in brain, we injected AAV-hfCas13x.1/cr9 into neonatal AS mice and observed that paternal UBE3A protein expression reached 35.9% in cortex and 41.6% in hippocampus of AS mice at 4 weeks post infection with AAV-hfCas13x.1/cr9 (Fig. 1C, D), compared with that in WT mice. We analyzed these results of the two genders separately. Obesity in female AS mice treated with hfCas13x.1/cr9 was significantly relieved (Fig. 1E), while male AS mice only showed slight trend of improvement, indicating a sex-specific effect, which was also observed in a previous study using SaCas9 to treat AS. In addition, AS mice treated with hfCas13x.1/cr9 also showed improvement in dowel test (Fig. 1G) and beam walking test (Fig. 1H). **Conclusions:** We demonstrated that RNA targeting of paternal Ube3a-ATS with the high-fidelity CRISPR-hfCas13x.1 system (Fig. 1A) could significantly restore Ube3a expression in the cultured mouse neurons and *in vivo*, reduce obesity and improve several behavioral deficits in AS mice, providing a potential and promising therapeutic strategy for patients with AS.

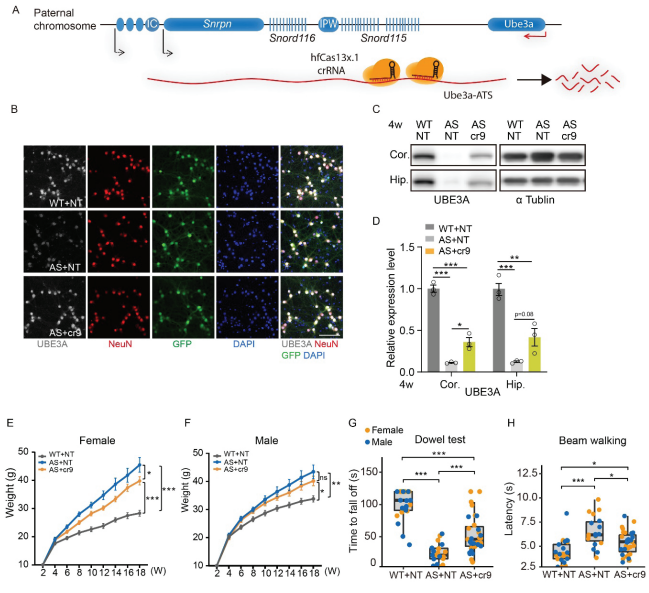

Fig. 1. The treatment effect of AAV-hfCas13X/cr9 in AS mice. A, Schematic diagram of the Ube3a locus in the mouse genome. **B**, Immunofluorescence staining for indicated proteins in lentivirus-infected primary neurons of AS mice. Primary neurons of WT or AS mice were infected with lentivirus containing hfCas13x.1/U6-NT or hfCas13x.1/U6-cr9. **C**, **D**, Western blot (**C**) and quantification (**D**) of protein expression in the cerebral cortex (cor.) and hippocampus (hip.) of WT and AS mice with indicated treatment at 4 weeks. **E**, **F**, Body weight. **G**, **H**, Dowel test and beam walking data in mice with indicated treatment.

1625 Deciphering the Mechanism of Nuclease-Free Homologous Recombination Based Genome Editing

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The replication-defective, non-pathogenic, adeno-associated viruses (AAVs) have gained in prominence for their therapeutic potential in genetic medicine. One unique property of AAV is their exclusive ability to mediate homologous recombination (HR) in the absence of exogenous nucleases. The stem cell-derived AAVHSCs in particular have a propensity to mediate precise and efficient HR with high on-target accuracy at significantly higher efficiencies than other AAV serotypes. The editing outcomes achieved by AAVHSC are precise without accompanying indel mutations or insertion of viral sequences, including ITRs at the edited genomic loci. In addition, the absence of a requirement for prior nuclease-mediated cleavage preserves genome-wide integrity. Notably, AAVHSC editing occurs efficiently in vivo in post-mitotic cells and tissues. In addition, AAV has the clear advantage of a built-in delivery mechanism. Importantly, the significantly higher in vivo and in vitro editing efficiencies displayed by AAVHSC editing vectors enables therapeutic translation. We have previously shown that the presence of functional BRCA2 is obligatory for successful AAVHSC-mediated HR. Other absolute requirements for effective AAVHSC genome editing include use of single stranded editing genomes which are bounded at the 5' and 3' ends by the palindromic self-complementary inverted terminal repeats (ITRs) which self-base pair to form T or U-shaped structures. In addition, the single-stranded AAV genomes must encode homologous sequences complementary to the target chromosomal loci to be edited. However, little is known about the exact mechanisms involved. To delineate the mechanism of AAV-mediated HR, we hypothesized that the transition between the single-stranded AAV editing genomes and the self-complementary palindromic AAV ITRs forms a unique structure that resembles a stalled replication fork. This junction is then recognized by proteins of the HR complex which localize to the transition site on the AAV editing genome. This in turn leads to the onset of HR. To test this hypothesis, we evaluated the direct interaction of cellular HR proteins with the AAVHSC editing genome. Primary human cells were transduced with packaged and purified AAVHSC editing vectors. Intranuclear AAVHSC editing genomes were then analyzed for their interaction with a panel of HR-associated and DNA repair proteins by chromatin immunoprecipitation (ChIP) analyses at different time points post-transduction. ChIP analyses revealed the preferential localization of HR proteins including BRCA2, Rad51 and Rad52 to the AAV editing genomes. Further mapping demonstrated that binding of BRCA2 in particular occurred near the ITRs. Electrophoretic mobility shift analyses were then performed to independently confirm the ChIP data. Results showed specific binding of BRCA2, Rad51 and Rad 52 to AAV ITRs. In conclusion, our results demonstrated that transduction with AAVHSC editing vectors specifically and preferentially recruited cellular HR proteins, including BRCA2 to the single stranded AAV editing genomes which then led to the onset of HR.

1626 Effects of Transgene Size on Self-Complementary rAAV Vectors

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Recombinant adeno-associated virus (rAAV) vectors are a promising tool for gene therapy due to their ability to efficiently and stably deliver genetic material to target cells. rAAV genomes can be packaged either in single-stranded DNA format (ssAAV) or a double-stranded, self-complementary DNA format (scAAV). Due to circumvention of the second-strand synthesis step required for transgene expression, scAAV vectors typically support higher and faster expression compared to ssAAV vectors. However, this enhanced expression comes at the cost of a reduced genome capacity (approximately 2.3 kb ssDNA vs 4.7 kb ssDNA for ssAAV designs). Manufacture of rAAV vectors at the scale required for addressing patient need remains a significant challenge. The baculovirus-infected Sf9 insect cell system is an attractive option to address the problem of scalability. However, the impact of transgene size for rAAV genomes has not been explored in the context of this system. We therefore sought to investigate the effect of transgene size on the functionality and physical characteristics of scAAV vectors produced in both HEK293-plasmid and Sf9-baculovirus systems. We constructed scAAV vectors of different sizes ranging from 2.7 kb to 5.4 kb ssDNA and produced these vectors in both the Sf9-baculovirus and HEK293 triple-plasmid transfection system. We characterized these vector preparations for productivity, vector genome heterogeneity, and transduction efficiency. Our results suggest that transgene size moderately impacts productivity of the vector in HEK293-plasmid systems but not in the Sf9-baculovirus system. Furthermore, we observed that the size of the transgene also affected vector genome heterogeneity in a production system-specific manner. As transgene size increased, the single predominant band was lost at a smaller threshold in Sf9-baculovirus compared to HEK293. In conclusion, our study demonstrates that transgene size of scAAV vectors affect

transduction efficiency, and this impact is dependent on expression system used to generate the vector. These findings have important implications for the development of scAAV vectors for gene therapy applications, as the size of the transgene must be carefully considered to ensure optimal vector performance. Future studies are being done to identify the optimal transgene size that would enable manufacturing of high-quality scAAV vectors.

1627 Integrated Purification Process Development Strategy to Enable Robust and Scalable Manufacturing of AAV

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Recombinant adeno-associated virus (rAAV) based vector is the most prominent modality used in in vivo gene therapy, due to its favorable profile in safety, efficacy, and durability. However, the manufacturing of rAAV vectors remains limiting. Rapid process development cycles that lead to robust and scalable manufacturing process is a prerequisite, to enable fast to clinic and reliable supply with consistent product quality for commercialization. Current challenges in downstream manufacturing process design include 1. processing bottle neck created by large volume low titer bioreactor harvest 2. low percentages of fully packaged vectors and often highly complex compositions of empty and partial capsids in feed stream 3. product and process related impurities 4. heightened regulatory expectations on viral clearance 5. scale up and scale down differences that may impact product quality and productivity 6. high production cost 7. limited material supply for process development. Several approaches can help address above challenges: 1. Reconfigure equipment to enable aggressive scale-down to micro-scale for process development. 2. Select high throughput, quick turnaround assays for efficient development cycle. 3. Leverage mechanistic modeling and process modeling to gain process understanding. 4. Employ high throughput automation and Design of Experiment (DoE) to enable process robustness evaluation. 5. Understand scale up differences that can present product quality or yield impact, and mitigate risks through targeted manufacturing equipment testing or pilot studies. We will present a comprehensive process development and process characterization strategy, with case studies demonstrating how to overcome key downstream purification challenges, particularly in empty, partial, and full capsid separation. We will also highlight areas of opportunity to enable future high efficiency AAV purification platform in anticipation of significant upstream viral vector production enhancement.

1628 Cryptic Splice Products in *PSEN2* Represent Novel Therapeutic Targets for Alzheimer's Disease

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Pathogenic variants in *APP*, *PSEN1* and *PSEN2* can cause early-onset familial Alzheimer's disease (AD). The molecular events leading to AD in individuals with late-onset sporadic AD are still unclear even though they share the same clinical and pathological features of AD. Using targeted PacBio long read isoform sequencing (Iso-Seq), we recently reported the characterization of tens of thousands of complete *PSEN1* and *PSEN2* transcripts in the prefrontal cortex of individuals with sporadic AD, familial AD (carrying *PSEN1* and *PSEN2* pathogenic variants), and controls. Surprisingly, we found several alternative splicing differences in *PSEN2* that are significantly elevated in sporadic AD including inclusion of a human-specific cryptic exon present in intron 9 of *PSEN2* (termed exon 9B) present in 11.8% of full-length reads in sporadic AD samples relative to 1.9% of controls ($p = 0.002$) and 3.4% of *PSEN* variant carriers ($p = 0.005$) as well as a 77bp intron retention product prior to exon 6. These *PSEN2* splice products are predicted to generate a prematurely truncated PSEN2 protein and were confirmed in an independent RNA-seq dataset of ~80 AD cases and ~80 controls from cerebellum tissue (Course et al., Brain 2022). To extend these findings we analyzed *PSEN2* exon 9B isoform levels in additional brain regions. We found exon 9B was significantly elevated in sporadic AD relative to controls in the parietal lobe ($p = 0.02$) as well as the temporal lobe ($p = <0.001$). However, levels of exon 9B were independent of pathology associated with the RNA binding proteins TDP-43 or MSUT2. We are testing functional effects of exon 9B inclusion in Aβ production and PSEN2 protein levels and utilizing antisense oligonucleotide approaches to target these aberrant isoforms. Collectively, our findings expand our understanding of *PSEN2* splice variants in the development of late-onset sporadic AD thereby revealing novel potential therapeutic strategies for AD.

1629 Gene Therapy Development for DDX3X Syndrome

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DDX3X syndrome is a neurodevelopmental disorder first described in 2015 and today is thought to be one of the leading known genetic causes of intellectual disability in females. The syndrome is caused by mutations in the Dead-Box Helicase 3 X-Linked gene (*DDX3X*), an abundant and ubiquitously expressed RNA helicase. Despite the clinical burden of DDX3X syndrome and the known genetic cause, no study to date has determined whether gene-based therapies would be an effective treatment for these patients. Our hypothesis is that DDX3X gene reinstatement, after altered DDX3X function during prenatal development, will restore proper protein function and, therefore,

ameliorate disease symptoms preclinically in a timepoint dependent manner that is sensitive to the level of DDX3X. To initially determine preclinically if gene replacement therapy for DDX3X syndrome is beneficial, we have developed an AAV9 gene therapy carrying a functional copy of *DDX3X* and are testing its safety and efficacy in mice. The mouse model used in our study is a previously characterized haploinsufficient *Ddx3x* (*Ddx3x*$^{+/-}$) mouse with construct and face validity for DDX3X syndrome. *Ddx3x*$^{+/-}$ females and *Ddx3x*$^{flox/+}$ control female littermates were injected at postnatal day (P) 1 via a bilateral intracerebroventricular injection with vehicle or AAV9-DDX3X at a low or high dose. To determine efficacy and safety, the mice are assessed behaviorally for 1 year. Developmental milestones, spanning physical, sensory, and motor domains are assessed from P2-20. Anxiety, EEG spike trains, and sleep behavior are assessed at 3-4 months of age. Anxiety and motor function is assessed at 12 months, followed by a full necropsy for histological studies. *Ddx3x*$^{+/-}$ females treated with low and high dose AAV9 have a trend towards less falls on a negative geotaxis paradigm compared to *Ddx3x*$^{+/-}$ females treated with vehicle in the first 3 weeks of life, but no other benefit was noted from the P2-20 developmental assessments. *Ddx3x*$^{flox/+}$ control female littermates treated with low or high dose AAV9 do not show any behavioral toxicity compared to *Ddx3x*$^{flox/+}$ females treated with vehicle on these developmental milestones. *Ddx3x*$^{+/-}$ females treated with high dose, but not low dose, have significantly reduced anxiety compared to *Ddx3x*$^{+/-}$ females treated with vehicle at 3 months, suggesting that there is a threshold for the amount of DDX3X needed for therapeutic benefit. However, *Ddx3x*$^{flox/+}$ control female littermates treated with high dose AAV9 have increased anxiety, suggesting that overexpressing DDX3X to this level can cause behavioral changes. Sustained anxiety behavior changes will be assessed at 12 months. Our results indicate that the dosage of DDX3X is important for behavior, where not enough or too much may lead to altered behavior. Determining the optimal amount of DDX3X expression for normal development and function will be assessed in future work and help guide designing the proper gene therapy vector to treat DDX3X syndrome.

1630 A New Method to Design Lineage Specific Promoters Enables Innovative Immunotherapy Approach Using CAR in Hematopoietic Stem Cells

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The growing fields of stem-cell-derived therapy for cell and gene therapy are currently facing an important bottleneck: the impossibility to target the expression of a gene into a given cell or tissue when a gene is modified in stem cells. This bottleneck currently prevents the possibility of using modified stem cells in many therapeutic situations. There is a need for tools to induce targeted gene expression in selected cells, both in terms of localization (tissue) and timing (stage of differentiation). To overcome the issue, we have developed a bioinformatic pipeline to identify key non-coding regions of the genome that have enhancer properties and can act as progeny or lineage-specific promoters when coupled with minimal promoter elements. To demonstrate the versatility of our *in silico* method we have applied it to both hematopoietic lineages, by designing T and/or NK-specific synthetic promoters, and non-hematopoietic lineages by generating muscle-specific promoter. Synthetic promoter specificity was validated using either hematopoietic stem cells (HSCs) or induced pluripotent stem cells (IPSCs) transduced with lentiviral vectors coding Green Fluorescent Protein (GFP) reporter gene under synthetic promoters. Modified HSCs were differentiated *in vitro*, using an organoid thymic differentiation system, into T cells, NK cells and B cells. Lead candidates were confirmed *in vivo* in the Bone Marrow Liver Thymus (BLT) humanized mouse model. Both *in vitro* and *in vivo*, GFP expression was observed only in T and NK subpopulations after HSC transduction with a T/NK-specific promoter. Modified IPSCs were differentiated into the three primary germ layers (mesoderm, endoderm and ectoderm) and then into neurons, hepatocytes and muscle lineages. Flow cytometry and immunofluorescence results showed that our muscle-specific promoter induced GFP expression only in mature terminally differentiated myotubes. GFP expression was observed neither in muscle cell progenitors, nor in earlier stages of differentiation or in neural or hepatic lineages. To demonstrate the potential of our synthetic promoter design platform, we used our technology to develop HSC-based chimeric antigen receptor (CAR) therapy for the treatment of refractory cancer. Despite the high efficacy of CAR-T therapy, many patients do not respond or will relapse after an initial response, in part due to low *in vivo* persistence of engineered T cells, poor proliferation and T-cell exhaustion. To address these issues, we explored an alternative therapeutic approach in which CAR transduction is made in HSCs, instead of T cells, allowing a continuous and progressive replenishment of CAR-modified cells. Constitutive expression of a CAR in the whole hematopoietic compartment highly increases the risk of disrupting cell differentiation process and activating a proto-oncogene. We showed in the BLT humanized mice that high level of CAR expression on T cell progenitors strongly impair T cell development. HSCs engineered with a CAR under human elongation factor-1 (EF1)-α constitutive promoter failed in differentiating into mature T cells. By substituting the EF1-α promoter for our synthetic T/NK-bispecific promoter in the CAR cassette, we overcome *in vitro* CAR-T cell differentiation blocking and generate simple positive mature CD4 and CD8 CAR-T cells derived from CAR-engineered HSCs. Demonstrating the validity and the functionality of our specific promoters represents a major advance in stem-cell-derived therapy, and an example of a promotor design method that could be applicable to many other fundamental and applicated research fields.

1631 Gene Therapy Mediated Transdifferentiation of Astrocytes to Neurons for the Treatment of Parkinson's Disease

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In situ cell reprogramming offers the hope of a regenerative therapeutic approach to numerous neurodegenerative diseases. Using AAVs to deliver reprogramming factors to glial cells can result in the transdifferentiation of these cells into neurons and the replenishment

of specific neuronal cell populations lost in a given disorder. Parkinson's disease (PD) results in the loss of dopaminergic neurons in the substantia nigra and decreased dopamine content within the striatum. While gene therapies for monogenetic forms of PD are in development, these do not address the unmet need of the majority of patients whose PD is idiopathic. *In situ* cell programming offers a gene-agnostic approach to address the large patient population without a known genetic cause. Our investigational therapeutic is an AAV vector encoding a reprogramming factor under the control of a glia-specific promoter. In a chemically induced mouse model of PD, treatment with this AAV led to the transdifferentiation of glial cells into electrophysiologically active neurons that express markers of mature dopamine neurons and are capable of taking up and releasing dopamine. Further, this treatment alleviated multiple motor defects common in this PD mouse model. In a chemically induced non-human primate model of PD, treatment with our investigational therapeutic led to improved motor behavior, increased dopamine concentration in the CSF, and enhanced dopamine signal within the striatum as measured by PET-CT. These effects were persistent out to at least one-year post-treatment. These animals continue to be observed two and a half years post-administration, and additional data are expected. Together, these findings support the promising therapeutic potential of *in situ* cell reprogramming for PD and set the stage for testing this novel therapeutic approach in a broad range of neurodegenerative conditions.

1632 Awake Intracerebroventricular Delivery and Safety Assessment of Oligonucleotides in a Large Animal Model

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Background: Oligonucleotide therapeutics, including small inhibitory RNAs (siRNAs) or antisense oligonucleotides (ASOs) offer promise in the treatment of several neurological conditions by knocking down expression of toxic proteins and enhancing expression of loss of function proteins (ex. splicing mutations). This class of therapeutics offers great promise in the treatment of previously untreatable disorders; however, they carry a well-established dose related acute toxicity (generalized and myoclonic seizures) at the time of administration. These seizures are hypothesized to be due to chelation of cations by oligonucleotides in the extracellular fluid, thereby lowering the resting membrane potential. Formulation of these compounds in cations has been postulated to prevent the acute toxicity, but a sensitive and quantitative way to track vacant or minor seizure activity in formulation development is lacking. For this reason, we developed a method to track seizure activity in awake animals during and for 24-hours after injection. **Methods:** Here we describe a new surgical technique for intracerebroventricular (ICV) delivery of neurotherapeutics. A catheter was placed into the lateral ventricle of sheep using a combination of pre-operative MRI- and post operative based neuronavigation. The catheter was fixed utilizing a 3D printed anchor system consisting of plastic and ceramic parts (Fig 1). The catheter is then connected to an MRI-safe reservoir with the incision closed over the apparatus. Days to weeks later, a wireless Holter electroencephalography (EEG) is used to measure brain activity and seizure activity is recorded (TrackIt System). The oligonucleotide is the injected under dexmedetomidine sedation and immediately reversed after for EEG recordings after which the animal is returned to its pen with electrodes in place and monitored by EEG and video for the next 24 hours. **Results:** Our surgical technique has reliably fixed the ICV catheter in place in sheep allowed normal outdoor activity for the duration of the study. We are reliably able to detect gamma, beta alpha theta and delta waves for a 24-hour period. EEG abnormalities associated with oligonucleotide administration and prevention with formulation is detectable by EEG in a dose dependent manner. The changes in waveform correlate with alterations in animal behavior phenotypes. Spectral quantification of EEG is underway.

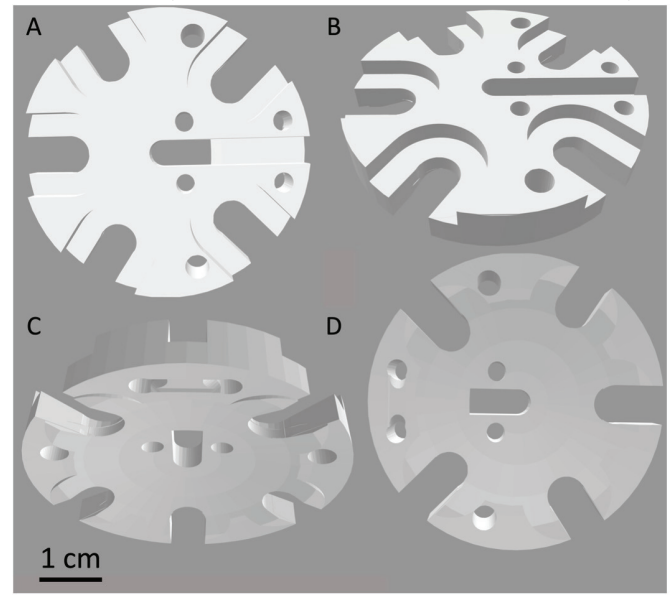

Figure 1. Prototype

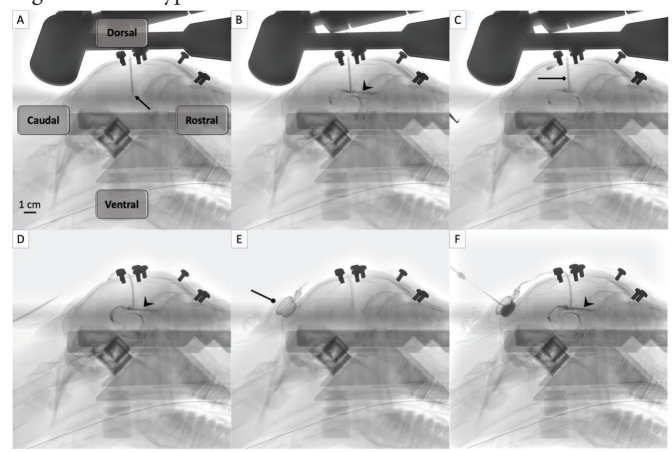

Figure 2. Sequenced intraoperative lateral x-ray images of ICV catheter placement

1633 Endosomal Escape Vehicle-Oligonucleotide Conjugates for the Targeted Upregulation and Downregulation of Gene Expression

Ziqing Leo Qian, Xiang Li, Xiulong Shen, Mahboubeh Kheirabadi, Amy N. Hicks, Mark Wysk, Haoming Liu, Ajay Kumar, Nelsa L. Estrella, Patrick G. Dougherty, Suresh Peddigari, Anushree Pathak, Kimberli J. Kamer, Wenlong Lian, Nanjun Liu, Matthew Streeter, Sara L. Blake, Christopher M. Brennan, Ning Li, Vlad Batagui, Keyede Oye, Ningguo Gao, Danel Wang, Arianna Bonilla, Mohanraj Dhanabal, Mahasweta Girgenrath, Natarajan Sethuraman

Entrada Therapeutics, Boston, MA

To overcome current limitations of oligonucleotide therapeutic delivery, we have designed a family of proprietary cyclic CPPs that form the core of our Endosomal Escape Vehicle (EEV™) technology covalently conjugated to oligonucleotides. Through screening a library of EEVs, we identified EEVs for functional delivery to target cell and tissue types. We employed our EEV-PMO (phosphorodiamidate morpholino oligomer) technology in Duchenne muscular dystrophy (DMD) preclinical models and evaluated the ability of our EEV-PMO conjugates to upregulate specific gene expression via exon skipping. In DMD, mutations result in a reading frame shift causing a lack of functional dystrophin. In mice harboring a *DMD* exon 23 nonsense mutation, we showed that monthly doses of an exon 23-skipping EEV-PMO resulted in restoration of dystrophin protein in skeletal and cardiac muscle. Next, we applied our EEV-PMO approach to down-regulate interferon regulatory factor 5 (IRF5) gene expression. Overexpression of IRF5 is associated with inflammatory and autoimmune diseases. To knockdown IRF5, we identified steric blocking oligonucleotides that modulate splicing, promote out-of-frame shift, and knockdown *IRF5* mRNA level. Treatment with an IRF5-targeting EEV-PMO resulted in potent and dose-dependent knockdown of IRF5 *in vitro* and *in vivo*, indicating the applicability of our EEV-PMO approach in modulating gene expression in immune cells. These results demonstrate the ability of our EEV platform to efficiently deliver oligonucleotides to specific target cell and tissue types. In addition, our approach has broad applicability to up- or down-regulate target gene expression through distinct mechanisms of action.

1634 Optimization of a Suicide Gene Vector for the GEN2 Cancer Immunotherapy Clinical Trial

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The optimization of a suicide gene which serves to unmask neoantigens within tumor cells and generate immune activation was undertaken using a series of criteria focused on more effective distribution of the suicide gene within the tumor cells and better selectivity for the prodrug, ganciclovir (GCV). GEN2 is a highly engineered non-replicating Moloney Murine Leukemia Viral (MoMLV) vector encoding for a modified Herpes Simplex Virus Thymidine Kinase (HSV-TK) suicide gene. HSV-TK is an enzyme that locally converts an orally administered prodrug activator (valganciclovir) into a toxic cellular metabolite that ultimately induces cell death in transduced and nearby cancer cells, release of neoantigens into the tumor microenvironment and activation of local and system immune responses. These responses include increased uptake of tumor cell antigens by dendritic cells, maturation of various other antigen presenting cells for enhanced antigen capture, more cross-priming of T cells, and increased tumor infiltration by, and activation of, cytotoxic T cells. In order to enhance the cell killing activity of HSV-TK, three strategies were used. 1. The distribution of HSV-TK was enhanced in the cytosol of the targeted cancer cells by: a. modification of nuclear localization signal (NLS) which results in uniform distribution of HSV-TK in the nucleus and cytosol; b. insertion of a nuclear export signal (NES) at the 5' terminus of the HSV-TK sequence to relocate HSV-TK into the cytoplasm. Distribution of the HSV-TK enzyme to the cytosol enhances the cellular efficiency of conversion of the GCV to its active toxic metabolite and also the availability of this metabolite to affect surrounding cancer cells through bystander effect. 2. Increasing selectivity of HSV-TK for GCV over thymidine was achieved through the mutation of the substrate binding domain. 3. The optimization of the coding sequence for the HSV-TK was achieved through: a. codon optimization to remove rare and low use codons to improve HSV-TK protein expression; b. lowered GC content within the newly codon optimized gene to avoid gene synthesis and other gene expression problems; c. removal of known splice acceptor and splice donor sequences within the optimized gene along removal of polypyrimidine tracts that may be involved in splicing; d. to further optimize expression of HSV-TK protein, a strong Kozak translation initiation sequence was introduced in front of the start codon while removing possible Kozak sequences within HSV-TK open reading frame. Overall, the combination of all modifications renders GEN2 more potent in cancer cell killing compared to all other vectors tested. GEN2 (HSV-TK-GMCSF) is currently being evaluated in a Phase I clinical trial.

1635 TROCEPT-01: A Tumor Targeted Oncolytic Adenovirus Vector for Delivery of Immunotherapeutic Payloads by Intravenous Administration

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Background Immune checkpoint inhibitors (ICIs) have revolutionized immunotherapy, but efficacy and response rates are limited, dependent on the ability of patient T cells to recognize and activate against tumor antigens. Additionally, systemic delivery can lead to dose limiting toxicity. These, and other treatment modalities, would benefit from efficient localized tumor delivery to minimize off-target toxicity and maximize efficacy at the point of need. TROCEPT is a novel tumor-selective delivery technology, based on adenovirus, engineered to

prevent sequestration in normal tissues. Removal of normal tissue tropisms addresses the main limitation of other viral therapies: hepatic sequestration. TROCEPT is further engineered to specifically bind to a tumor marker, αvβ6 integrin, expressed on most carcinomas, and carry transgenes encoding immune checkpoint inhibitors for in-tumor delivery. Once delivered, this results in a function ICI being concentrated at the point of need. Thus, TROCEPT-01 enables tumor-localized generation of ICI payloads. In this study we demonstrate that TROCEPT-01 overcomes these limitations,to deliver ICI's specifically to tumors, by I.V. administration, without off target sequestration, at high titers. **Methods** TROCEPT-01 was rationally engineered using recombineering. αvβ6 integrin-dependent cell entry of TROCEPT-01, production of ICIs in multiple tumor cell lines, and functionality of *in-tumor* generated ICI was confirmed *in vitro*. Finally, a murine model engrafted with a human αvβ6 integrin-positive tumor, was used to assess biodistribution of TROCEPT-01, after intravenous delivery, using a bioluminescence *in vivo* imaging system and qPCR. **Results** *In vitro* testing confirms TROCEPT-01's exquisite selectivity for tumor cells expressing αvβ6 and ablation of native viral tropisms. We show high titer expression of functional ICI's in cancer cell lines. *In vivo* experiments demonstrate TROCEPT-01 is well tolerated and it's biodistribution is tumor-localized, with high concentrations of ICI in the tumor and low concentrations of ICI detected in peripheral blood. **Conclusions** TROCEPT displays an improved biodistribution profile compared to other I.V. oncolytic viral vectors. It's tumor selective transgene delivery and production of ICIs enables high titer local dosing of the tumor, addressing systemic toxicity and potentially increasing efficacy. Delivery of ICI's via TROCEPT has the potential for synergistic cancer cell killing by expressing viral associated antigens and cell lysis, driving immune cell infiltration, while breaking tumor immune tolerance. Reduced off target infection enables a greater proportion of the therapy to reach the tumor than other oncolytic virus approaches which rely on non-specific cell entry. Therefore, TROCEPT represents an exciting next-generation viral vectored immunotherapy for αvβ6 positive cancers.

1636 VE-Cadherin Gene Editing Shows a Kaiso-Dependent Loss of Vasculogenic Mimicry in Uveal Melanoma Cells

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Solid tumors require blood vessels for growth, and access to oxygen and nutrients, and anti-angiogenic therapies are designed to target vascular endothelial cells (ECs) to form tumor blood vessels. Although numerous preclinical models have recognized the efficient use of angiogenesis inhibitors to limit tumor growth, collectively only a growth delay has been achieved in the clinic. In 1999, Maniotis et al. presented a new interpretation of previous findings describing cancer cells covering non-endothelial vascular channels that contained red blood cells. This initial report defined tumor cell Vasculogenic Mimicry (VM) as the de novo formation of perfused, matrix-rich, vasculogenic-like network in a three-dimensional (3D) matrix in vitro, which resembled the matrix-rich network observed in aggressive tumors in patients. The presence of VM is associated with a high tumor grade, short survival, invasion, and metastasis. ECs express various members of the cadherin superfamily, in particular vascular endothelial (VE-) cadherin (VEC), which is the main adhesion receptor of endothelial adherent junctions. Aberrant extra-vascular expression of VEC has been observed in certain cancer types associated with VM. VEC is a trans-membrane protein commonly expressed in the endothelium, where it is responsible for cell-cell adhesion. Although VEC used to be considered specific for ECs, its expression has been strongly associated with aggressiveness and VM in melanoma. In the current study, we have elucidated the mechanistic link leading from elevated accumulation of phosphorylated VEC (pVEC) to induction of VM in metastatic uveal melanoma (UM). Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase co-activated by integrin and VEGFR-2 receptors in the control of vascular permeability. Recent studies have shown that the VECY658 residue prevents targets FAK in tumour-associated ECs, and endothelial FAK activity inhibits tumor metastasis by enhancing barrier function. Using a co-immunoprecipitation approach, we show that VEC forms a complex with p120 and the transcriptional repressor kaiso into the nucleus. Abrogation of VEC phosphorylation, through FAK inhibition (PF-271), diminished complex formation and its nuclear import. Interestingly, the pVEC (Y658) depletion forced the accumulation of VEC and p120 in the cytoplasmic fraction. In addition, aberrant extravascular expression of VE-cadherin has been observed in certain MV-associated cancers. Gene-edited MUM2B uveal melanoma cells, deficient in VE-cadherin by CRISPR/Cas9 technology, show loss of the ability to develop VM. Moreover, rescue of wild-type VE-cadherin reversed the ability to form MV; conversely, expression of VE-cadherin Y658F blunted MV in vitro. On the other hand, Kaiso is a member of the BTB/POZ subfamily of zinc finger proteins (POZ-ZF); which recognizes and binds a specific DNA consensus sequence (kaiso binding sites KBS, TCCTGCnA, as a transcriptional repressor). Previous studies have shown that kaiso forms a complex with p120, which prevents kaiso binding to its target genes promoters such as CCDN1, and WNT 11or MMP-7 among others. Chromatin Immunoprecipitation Assays (ChIP) reveal that both FAK and VEC silencing robustly increased kaiso binding to the WNT11 and CCND1 promoters and then negatively regulated kaiso-repressor activity, which resulted in abrogation of VM in vitro. These results establish a molecular paradigm associating pVEC with VM transformation of uveal melanoma cells owing to its ability to modulate p120 nuclear localization and kaiso-dependent gene expression.

1637 A S-HUDEP2 Reporter Cell Model to Monitor HBG Regulation for Treating Sickle Cell Disease

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Sickle cell disease (SCD) is an inherited hemoglobinopathy caused by a single point mutation in the β-globin gene (*HBB*), which leads to sickle hemoglobin (HbS) instead of adult hemoglobin (HbA) and causes severe pain, end organ damage and early mortality. CRISPR/Cas9 based gene-editing for treating SCD has been developed to induce fetal hemoglobin (HbF) which is a major hemoglobin in the

fetus. Although HbF is suppressed after birth, inducing an elevated HbF level by gene editing has shown promise to reduce morbidity and mortality among SCD patients. However, studies on HbF induction are limited by a conventional method to measure HbF level by intracellular staining followed by flow cytometry. The intracellular staining includes fixation of live cells, laborious sample preparation, and significant cell loss. These procedures damage DNA and hinder long range PCR, which is necessary to thoroughly investigate genetic modifications or large deletions that may contribute reactivation of HbF. We identified HbF induction by *HBB* cutting, but investigation on the mechanism behind it has been limited.Here, we established a fluorescent reporter cell model that enables monitoring HbF expression in living cells to overcome limitations from conventional intracellular staining. In Sickle-Human Umbilical cord Derived Erythroid Progenitor 2 (S-HUDEP2), which is an immortalized $CD34^+$ hematopoietic stem and progenitor cell line with a sickle mutation in the *HBB*, we integrated mTagBFP2 gene at the C-terminus of *HBG1/2* by delivering HiFi *Sp*Cas9/gRNA targeting the *HBG* stop codon and a double-stranded DNA donor encoding P2A-mTagBFP2-polyA. We established single cell clones and identified one clone, H6, that clearly shows changes in BFP expression according to changes in HbF expression. In H6, 13% of cells expressed BFP^+ and 7% of cells expressed $HbF(FITC)^+$ in expansion and it was increased to 45% BFP^+ and 49% $HbF(FITC)^+$ after erythroid differentiation. We used H6 to perform gene-editing by HiFi *Sp*Cas9/sgRNA targeting *BCL11A* erythroid enhancer and erythroid differentiated for 8 days to induce HbF. After editing and erythroid differentiation, % BFP^+ cells was increased to 66% and % $HbF(FITC)^+$ cells was increased to 75%. We performed additional gene-editing by HiFi *Sp*Cas9/sgRNA targeting sickle mutation in *HBB* and erythroid differentiated for 8 days to see if % BFP^+ cells can represent % HbF^+ cells with a high correlation. We found that 72% of cells expressed BFP^+ and 90% of cells expressed $HbF(FITC)^+$ after editing *HBB* and erythroid differentiation. We drew a correlation between % HbF^+ cells and % BFP^+ cells induced by erythroid differentiation, gene-editing in *BCL11A* erythroid enhancer and *HBB*, and confirmed a high correlation with R^2 of 0.95. This high correlation indicated H6 can precisely represent % HbF^+ induced cells by % BFP^+ cells. Next, we sorted BFP^{high} and BFP^- from *HBB* edited H6 and performed long-range PCR followed by fragmentation and barcoding for Illumina MiSeq next generation sequencing (NGS) to identify allele profiles of cells expressing high HbF. More large deletions (27.6%) were found in BFP^{high} than in BFP^- (18.5%) and bulk *HBB* edited H6 (19.9%). The high frequency of large deletions in BFP^{high} indicates that large deletions generated by *HBB* cutting may contribute to HbF induction.In this study, we were able to establish single cell clones for the HBG-BFP reporter cell model and showed that H6 can precisely represent % HbF^+ by % BFP^+. We further used the clones to investigate HbF induction by *HBB* cutting and showed a relation between large deletions in *HBB* and HbF induction.In conclusion, the S-HUDEP2 HBG-BFP cell model can easily monitor HbF expression by BFP, and it can be utilized in other studies such as mechanism studies or gene-editing screening for HbF induction.

1638 AAV9.BVES Delivery Improves Muscular Dystrophy and Arrhythmia in a Mouse Model of LGMDR25

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Limb-girdle muscular dystrophy type R25 (LGMDR25) is caused by recessive mutations in BVES (blood vessel epicardial substance), characterized by progressive muscular dystrophy with muscle weakness and defective cardiac rhythm conduction in patients. Currently, there is no cure for LGMDR25 patients. Here we report the efficacy and safety of recombinant adeno-associated virus 9 (AAV9)-mediated delivery of human BVES driven by a muscle-specific promoter MHCK7 (AAV9.BVES) in a newly established BVES-knockout (BVES-KO) mouse model. First, intramuscular injection of AAV9.BVES efficiently transduced and significantly improved skeletal muscle histopathology and function of BVES-KO mice. Next, systemic delivery of AAV9. BVES following intraperitoneal injection into neonatal BVES-KO mice resulted in body-wide transduction of skeletal and cardiac muscles, with dramatic improvement in body weight gain, muscle mass, muscle strength, and exercise performance regardless of gender. AAV9.BVES also significantly mitigated the histopathological features of muscular dystrophy. Moreover, the heart rate reduction was normalized in BVES-KO mice under exercise-induced stress following systemic AAV9. BVES delivery. Finally, intravenous AAV9.BVES administration into adult BVES-KO mice after the disease onset also resulted in substantial improvement in body weight, muscle mass, muscle contractility, and stress-induced heart rhythm abnormality. No obvious adverse effects were detected. Taken together, our results provide the proof-of-principle evidence to support the AAV9.BVES gene therapy for LGMDR25.

1639 Re-Development of Glybera: A Novel Adeno-Associated Virus Gene Therapy for the Treatment of Lipoprotein Lipase Deficiency

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Lipoprotein lipase deficiency (LPLD) is a monogenetic recessive disease caused by mutations within the lipoprotein lipase (LPL) gene, resulting in the production of a catalytically inactive protein. LPLD causes accumulation of chylomicrons within the blood that leads to severe hypertriglyceridemia and occurrence of life-threatening complications such as pancreatitis. We previously developed Alipogene Tiparvovec (Glybera), the first adeno-associated virus (AAV) gene replacement therapy product to receive regulatory approval in the western world for the treatment of LPLD. However, Glybera is no longer marketed due to economical considerations. The aim of this study was to develop a more efficacious and cost-effective AAV-based gene therapy formulation for the treatment of LPLD. Briefly, all novel AAV formulations, including Glybera, encoded either a luciferase transgene or a gain-of-function human LPL^{S447X} transgene. The transgene was driven by several ubiquitous and tissue specific promoters and packaged in several AAV serotypes. All AAV formulations were delivered intramuscularly (quadriceps) in adult mice with LPLD at either 1.0E+11 gc/kg (low dose) or 1.0E+12 gc/kg (high dose). Mice were followed for up to 80 days post-AAV treatment. Plasma lipemia, triglycerides and transgene expression were assessed throughout the study to determine therapeutic efficacy. Plasma biochemistry (liver enzymes), immune markers (cytokine array), tissue pathology and biodistrubtion were assessed at study endpoint. Our studies revealed a novel formulation, pVR59, that resulted in complete normalization of plasma triglycerides (>98% plasma triglyceride lowering from baseline) in LPLD mice at 10-fold lower doses compared to Glybera. Our biodistribution studies revealed prominent gene transfer within the injected skeletal muscle and liver following treatment with pVR59. Furthermore, pVR59 was found to be well tolerated with no systemic or localized toxicity. Our results collectively suggest that it is feasible to develop a superior AAV-based gene therapy product for the treatment of LPLD. The identification of a novel AAV formulation that is superior to Glybera at lower doses, when paired with recent advances in AAV manufacturing processes and scale-up, will translate to deliver a potent drug with increased safety and efficacy, at a lower manufacturing cost.

1640 Nanotein's NanoSpark Expands Stemlike T Cells

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Cellular therapies are the cancer treatment of the future. These therapeutics work by efficiently seeking cancer cells for targeted destruction, while leaving healthy cells intact. CAR-T cells are the most clinically and commercially advanced of anti-cancer cell therapies. While incredibly effective, they have faced multiple challenges including manufacturing failures, long manufacturing times, and short-lived or poor treatment outcomes. Nanotein's novel platform technology, NanoSpark, can help overcome these challenges by accelerating the speed of CAR-T expansions, enhancing expansion to grow more cells per treatment, and shifting the growth profile of CAR-T cultures to favor the most long-lived and clinically effective CAR-T cell sub-populations, the stem-like central memory T cells (T_{scm}). Here we show that Nanotein's NanoSpark STEM-T formulation achieves enhanced expansion of total live T cells, total live $CD8^+$ cells, and $CD8^+$ T_{scm} cells during 10-day expansions of donor peripheral blood T cells. Taken together, these results demonstrate that Nanotein's NanoSpark technology can quickly expand T cells to high numbers while maintaining the therapeutically desirable, less differentiated T_{scm} subpopulation in $CD8^+$ T cells.

1641 Correcting CPS1 Deficiency in Primary Human Hepatocytes *In Vivo* Using AAV Delivered Cell-Cycle Independent Genome Editing

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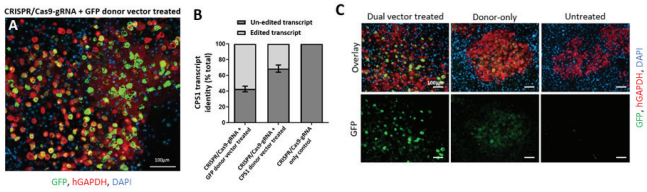
Background: Carbamoyl phosphate synthetase I (CPS1) deficiency is a rare genetic metabolic disorder that prevents or impairs the metabolism of blood ammonia. Loss of function of the CPS1 enzyme can cause neonatal death, or intellectual disability in surviving patients. The only treatment option for severe CPS1 deficiency is liver transplantation, however the disease is an ideal candidate for gene therapy. An effective and durable gene therapy for CPS1 deficiency must create permanent changes to the genome of hepatocytes such that therapeutic effects are maintained during paediatric liver growth. Additionally, repair must overcome a high threshold for therapeutic benefit that directly correlates to hepatic cell mass corrected. We aimed to develop a cell-cycle independent targeted genome editing approach to treat CPS1 deficiency in patient-derived primary human hepatocytes *in vivo*.
Methods: The $Fah^{-/-}$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (FRG) mouse model is immunodeficient and Fah negative, allowing for the engraftment and selective expansion of primary human hepatocytes. A dual

recombinant adeno-associated virus (rAAV) based approach was designed to deliver CRISPR/SaCas9 guided editing machinery and a promoter-less *GFP* or *CPS1* cDNA donor cassette for homology-independent genome insertion at the *CPS1* locus, under the native promoter. Both vectors were packaged in NP59 AAV capsid which is highly human hepatotropic. Wild-type (WT) human hepatocytes were engrafted into the FRG mouse and treated with vector ~11 weeks post engraftment at a dose within $1x10^{11}$vg/mouse to $5x10^{11}$vg/mouse. Vector treated mice were analyzed 6 weeks post injection. **Results:** A cohort of FRG mice engrafted with WT human hepatocytes (n=9) treated with $1x10^{11}$vg/mouse, $2.5x10^{11}$vg/mouse, or $5x10^{11}$vg/mouse of rAAV-NP95 CRISPR/SaCas9+gRNA vector only were analyzed for insertion/deletion (InDel) events at the Cas9 cut site. A dose of ≥800 vector genome copies per human diploid cell (via ddPCR), which correlated to $5x10^{11}$vg/mouse, was found to produce the maximum InDel events at the target loci. WT engrafted FRG mice treated with dual rAAV-CRISPR/SaCas9+gRNA vector and rAAV-*GFP* or -*CPS1* cDNA donor vectors, $5x10^{11}$vg/mouse of each vector, were analyzed for expression and genomic integration (n=4 per donor vector). Dual treated mice that received GFP donor vector showed GFP expression in up to 80% of human hepatocytes *in vivo* via immunofluorescence (Fig A). Successful integration of *GFP* or *CPS1* donor cDNA at the *CPS1* locus was observed in all dual-treated mice, as well as high levels of edited *CPS1* transcript compared to native transcript (Fig B). Thorough safety investigation of unwanted off-target events is currently under investigation. Mice treated with rAAV-*GFP* donor vector only at $5x10^{11}$vg/mouse show lowed levels of GFP expression compared to dual-treated mice, likely from off-target insertion (Fig C). **Conclusion:** These results validate our approach as a highly efficient gene integration system, able to target and edit a significant proportion of human hepatocytes *in vivo*. Our approach will be further investigated for phenotypic correction and safety in patient-derived CPS1-deficient primary human hepatocytes *in vivo*.



1642 Development of a Multi-Functional Peptide for Enhanced Efficacy of siRNA

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Glioblastoma Multiforme (GBM), a grade VI glioma, is an aggressive malignant tumor associated with a poor prognosis. With an incidence rate of 3.19 per 100,000, GBM has a median survival rate of 12-15 months and a five-year survival rate less than 5%. Typical treatments involve surgical removal, chemotherapy, and radiation; however, these result in low survival and harm to healthy cells. RNAi has demonstrated promise for treating many diseases through targeting specific mRNA to inhibit translation; however, significant barriers to delivery hinder the clinical translation. These barriers include non-specific delivery, insufficient cellular uptake, and lack of endosomal escape. Three classes of peptides have demonstrated the ability to overcome these specific barriers: targeting, cell-penetrating, and fusogenic peptides, respectively. Although these peptides have been studied individually, few studies have evaluated the effects of combining peptides to overcome multiple delivery barriers. Thus, this study aims to design a novel multifunctional peptide, or tandem peptide, for the delivery of siRNAs to treat GBM. Combining peptide sequences would provide synergy in their characteristics, developing more efficient nanocarriers. The tandem peptide combines a fusogenic peptide, 599, and EGFR-targeting peptide, GE11. A cationic tail creates a positive charge for electrostatic complexation with siRNA. STAT3, the target gene for RNAi, is an oncogene that plays a role in cell proliferation, differentiation, apoptosis, and angiogenesis in GBM. Therefore, it is hypothesized that the tandem peptide will enhance efficacious delivery of siRNA into glioblastoma cells, resulting in more effective STAT3 gene silencing compared to the fusogenic and targeting peptides alone. We have established the tandem peptide electrostatically complexes and protects free siRNA from degradation in mimicked physiological conditions, indicating stable complexes for clinical translatability. Characterization analysis of the peptide/siRNA complexes revealed formation of uniform, monodisperse, and positively charged nanoparticles (Figure 1a). These complex characteristics are favorable for delivery to cancer cells and interactions with the cellular membrane. Western Blots confirmed basal expression of the target receptor, EGFR, and the target oncogene, STAT3, in an array of GBM cell lines. A viability assay showed no difference in viability between cells treated with the peptide compared to untreated cells, indicating minimal toxicity. Fluorescent uptake imaging and flow cytometry showed the tandem peptide increased internalization of siRNA in GBM cells. To determine gene silencing capability, the tandem peptide was complexed with STAT3 siRNA and transfected into GBM cells. qPCR analysis shows the tandem peptide's ability to reduce the STAT3 mRNA, especially at a 60:1 ratio (Figure 1b). Future *in vitro* work will include confirming gene silencing through western blots and evaluating the downstream effect of STAT3 silencing through migration and invasion assays. Assays will be completed with the individual peptides as a comparison to evaluate the tandem peptide for improved delivery of bioactive siRNA. Future work will also focus on *in vivo* therapeutic potential of delivering tandem/siSTAT3 complexes for GBM.

(a)

Tandem Characterization		
Size	PDI	Zeta Potential
85.9 ± 1.82	0.117	12.4 ± 1.04

(b)

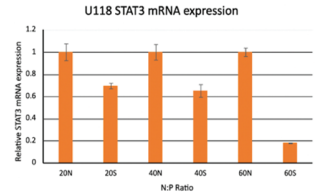

Figure 1. (a) Characterization of the tandem peptide complexed with siRNA at a 60:1 ratio through dynamic light scattering analysis. **(b)** qPCR analysis of STAT3 mRNA expression in U118 GBM cell line following transfection with tandem/siSTAT3 complexes

1643 Oncolytic Adenovirus Armed with Interferon Alpha Elicits Systemic Antitumor Immunity in Syrian Hamster Pancreatic Cancer Models

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Background: Pancreatic ductal adenocarcinoma (PDAC) remains a highly fatal disease due to an immunosuppressive tumor microenvironment. Our strategy focuses on the use of oncolytic adenoviruses (OAds) as cancer therapeutics. OAds are designed to selectively replicate and spread within a tumor, resulting in immunogenic cell death (ICD), which releases damage-associated molecular patterns, evoking strong antitumor immune responses. OAds are also capable of highly efficient delivery of immune-stimulating genes to cancer cells, achieving high concentrations of the gene products within the tumor. Here, we employed a replication competent OAd expressing syngeneic IFN-α (OAd-IFN), one of the most promising cytokines for combination strategies in cancer immunotherapy, and assessed its potential for therapeutic application in PDAC. **Methods and Results:** For this study, we developed novel immunological tools to assess the immunomodulatory role of OAd-IFN in immunocompetent Syrian hamsters. Unlike commonly used mouse models, Syrian hamsters are permissive to OAd replication, providing an advantage in predicting OAds behavior in humans. To identify the role of type I IFN in antitumor activity, we employed the identical luciferase-expressing control virus (OAd-Luc). We first evaluated the cytotoxicity of OAd-IFN and its gene production in hamster PDAC cell lines. OAd-IFN showed significantly higher cytotoxicity compared to OAd-Luc. OAd-IFN infected PDAC cells secreted IFN-α in a dose and time dependent manner, indicating efficient gene expression. Next, we examined inflammatory responses by quantitative RT-PCR, and found that a variety of proinflammatory chemokine genes, including CXCR3 ligands, and MHC I were upregulated in OAd-IFN infected cells compared to OAd-Luc infected or uninfected cells. The level of extracellular ATP measured by bioluminescent assay was also significantly increased in OAd-IFN infected cells, suggesting the potential of OAd-IFN to induce ICD. We then evaluated *in vivo* therapeutic and immunomodulatory activities of OAd-IFN. Syrian hamsters bearing HP-1 PDAC tumors in both flanks were treated intratumorally with OAd-IFN or OAd-Luc ($2.5x10^9$ pfu/injection), or PBS unilaterally every three days for a total of four injections. To evaluate the systemic efficacy ("abscopal effect") of OAd-IFN, the right flank tumor was left untreated. Compared to PBS and OAd-Luc treated groups, OAd-IFN showed significant tumor growth inhibition not only in the injected tumors, but also in the non-injected distant sites. Median survival was significantly improved in the OAd-IFN group (PBS: 27 days, OAd-Luc: 36 days, IFN-OAd: 40.5 days) without any adverse events. Importantly, we confirmed that, in this model, infectious viral particles (as quantified by $TCID_{50}$ assay) were restricted to only the injected sites, and were not responsible for the abscopal effect. These data suggest potential for OAd-IFN to induce direct oncolysis and systemic antitumor immunity *in vivo*. The underlying mechanisms of this phenomenon are currently being investigated using newly developed immunological assays for Syrian hamster models, including spatial transcriptome analysis. **Conclusion/Future directions:** This study demonstrated the potential of OAd-IFN to induce systemic antitumor immunity in immunocompetent OAd-permissive models, and will provide crucial knowledge for clinical translation of OAd-IFN-based therapies for PDAC.

1644 Developing Novel AAV Capsids for Ophthalmic Gene Therapy

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Gene delivery tools are the key for gene therapy for different diseases, including vision diseases. AAVs are often used to deliver genes to many different organs in basic and translational research. However, there are no effective AAVs to transduce the retina cells in an efficient and specific manner. Here, we modified the AAV capsids to create AAV libraries, and then we identified the potential AAVs with an in vivo animal screening strategy. In this study, we discovered a novel AAV variant, named as AAV-WM01, which can infect retinal pigment epithelium (RPE) cells efficiently in mouse retina by intravitreal injection. Further experiments indicated that AAV-WM01 specifically transduce RPE cells with high efficiency and specificity. Importantly, AAV-WM01 can deliver the gene for anti-VEGF to substantially alleviate the wAMD symdromes in the non-human primate animal model. Based on these exciting pre-clinical results, we are carrying out clinical studies to treat wAMD with AAV-WM01, and the clinical results are expected to release this year. Furthermore, we have found a couple of AAV variants can transduce other layer cells in the retina with high efficiency by intravitreal injection, which may hold the potential to treat other types of vision diseases.

1646 Intravenous Administration of AAV-GLA Corrects Substrate Level in the Mouse Model of Fabry Disease

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Fabry disease (FD) is a rare X-linked metabolic disorder caused by deficiency of lysosomal enzyme α-galactosidase A (α-Gal A) due to pathogenic mutations in the GLA gene.The progressive accumulation of substrates within lysosomes results in cellular dysfunction and multiple organ damage. The current enzyme replacement therapy is an effective treatment for FD, however, bi-weekly lifelong infusions are significant burdens for patients. To provide a single administration with long-lasting effects, we aim to develop a recombinant adeno-associated virus (rAAV) vector-based gene therapy for FD. The codon-optimized human GLA (hGLA) cDNA with minimal CpG was generated with two

algorithms. A series of synthetic hepatotropic promoters were evaluated *in vitro*. An optimized transgene expression cassette was then selected and packaged into AAV9 (scAAV9-hGLA). A single intravenous injection of scAAV9-hGLA to 10-week-old α-Gal A KO male mice resulted in dose-dependent α-Gal A activity in the serum, liver, heart and kidney. Stable expression of α-Gal A in serum sustained until the end of 8-week mouse study. Compared to wild type mice treated with vehicle control (buffer), the GLA-KO mice received the lowest dose (2×10^{11} vg/kg) showed 178-fold (×), 58×, 3×, 0.8× of α-Gal A activity in serum, liver, heart and kidney, respectively. The GLA-KO mice treated with 2×10^{12} vg/kg of scAAV9-hGLA showed 2360×, 1378×, 119×, 10× of α-Gal A activity. The highest dose (2×10^{13} vg/kg) resulted in up to 18,485× of α-Gal A activity in the serum. As lyso-Gb3 is a biomarker for FD, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify the substrate level. Compared to GLA-KO mice treated with vehicle control, all the AAV-treated GLA-KO mice showed significantly reduced lyso-Gb3 level in the serum. The 2×10^{11} vg/kg of scAAV9-hGLA treatment reduced the lyso-Gb3 to 2.5% of that of GLA-KO control mice. Three higher doses (2×10^{12}, 6×10^{12} and 2×10^{13} vg/kg) of treatment completely normalized the lyso-Gb3 level in the serum, liver, heart and kidneys. The codon-optimized hGLA transgene mRNA level correlated with AAV vector genome copies in the liver and showed a dose-dependent effect. Liver-derived robust human α-Gal A protein expression and proper glycosylation was demonstrated by western-blot. The immunostaining confirmed the presence of α-Gal A in the liver, heart and kidney. Histopathologic examination showed no observed toxicity with all the doses. Finally, we demonstrated that the effective dose can be further minimized by using an in-house developed novel liver-muscle tropic capsid AVT908 and promoter. Our data support development of low-dose AAV-GLA gene therapy for Fabry disease.

1647 IKC151V is a Bi-Cistronic Vector to Treat Retinal Conditions Associated with Significant Vascular Remodelling for the Treatment of Wet-AMD and Diabetic Retinopathy

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Purpose: Wet-form age-related macular degeneration (wAMD) and diabetic retinopathy (DR) are two of the most prevalent and debilitating retinal conditions and are associated with significant vascular remodelling, characterised by new blood vessel growth, vascular leakage, fibrosis, and degrees of inflammation. Frequently administered anti-VEGF therapeutics are the mainstay for the management of these conditions. Connective tissue growth factor (CTGF) is involved in vascular remodelling in both conditions and has been hypothesised to play a key role in the development of subretinal scars and capillary basal lamina thickening in wAMD and DR, respectively. We have designed a bi-cistronic AAV vector (IKC151V) that expresses a novel anti-VEGF component (VEGF-Capture2/VC2), with a modified Fc domain which reduces immune complex formation and transport into the systemic circulation, combined with a CTGF-attenuating component which is predicted to display greater efficacy in both eye diseases than anti-VEGF agents alone. **Methods:** The Fc-binding affinity of the recombinant novel VC2 protein was compared to aflibercept using ELISA-based assays. Null control vector (IKC166V), a vector expressing aflibercept alone (IKC163V) and IKC151V were administered bilaterally via the intravitreal route of delivery in the murine laser-induced CNV model. CNV leakage and lesion volume, were evaluated by fluorescence angiography (FA) and optical coherence tomography (OCT), whilst lesion area and transgene expression were evaluated via choroidal flatmounts and ELISA. In the *Vldlr-/-* knockout mouse model of CNV and subretinal fibrosis, IKC151V and IKC163V were administered intravitreally and neovascular leakage was assessed by FA. **Results:** The VC2 component in IKC151V displays reduced affinity for both human FcRn (IC_{50}: aflibercept= 34 nM, VC2= 24 microM) and Fc gamma-RI (IC_{50}: aflibercept= 20 pM, VC2= 63 microM). Using laser-induced CNV, IKC151V significantly reduced the lesion severity scores and lesion volume versus Null control vector at day 7, and performed better than the mono-cistronic aflibercept (IKC163V) 14 days after laser treatment (lesion volume (10^7 microns^3): Null= 1.66 ± 0.33, IKC163V= 0.80 ± 0.12*, IKC151V= 0.36 ± 0.08*; *p ≤0.001). The reduction in lesion size by IKC151V was greater than IKC163V despite significantly lower vitreous levels of anti-VEGF protein (IKC163V= 7.1 ± 3.2 microg/mL, IKC151V= 4.9 ± 1.7 microg/mL*; *p ≤0.01). Similarly, IKC151V treated *Vldlr-/-* mice demonstrated a reduced lesion severity score compared to IKC163V treated eyes (IKC163V= 0.79 ± 0.86, IKC151V= 0.35 ± 0.42) and a reduced total number of lesions (IKC163V= 0.39 ± 0.62, IKC151V= 0.0 ± 0.0). **Conclusion:** In IKC151V, the reduced affinity of VC2 for both FcRn and Fc gamma-RI receptors gives a greater safety potential over aflibercept. In two models of vascular remodelling, IKC151V was able to attenuate developing and existing neovascularisation to a greater extent than IKC163V expressing aflibercept alone, thus demonstrating an important role for CTGF in this pathology. These data support the therapeutic potential of IKC151V for the treatment of retinal conditions associated with significant vascular remodelling such as wAMD and DR.

1649 Characterization of Genomic Instability in Hematopoietic Stem Cells of *Blm*$^{tm3Brd/tm3Brd}$ Mice for Pre-Clinical Trial of Lentiviral Gene Transfer to Prevent Bloom Syndrome Related Hematological Malignancy

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Introduction: Bloom syndrome is a monogenic disease caused by the *BLM* gene mutation that leads to genomic instability and a predisposition to cancer. Some of the main characteristics of this genetic disorder are elevated sister chromatid exchange (SCE) and hypersensitivity to inter-strand cross-linking agents, such as mitomycin C (MMC), leading to the development of hematological malignancies. As patients do not have tolerance for cytotoxic chemotherapy or radiotherapy, hematological malignancies often result in a refractory clinical course and a poor prognosis. To address these issues, we plan to establish a preclinical trial of lentiviral gene transfer to hematopoietic stem cells (HSCs) to prevent the development of hematological malignancies in patients with Bloom syndrome. Although various mouse models with *Blm*-mutant alleles do not survive to term, *Blm*$^{tm3Brd/tm3Brd}$ (*Blm*$^{m3/m3}$) mice have been reported as a viable and fertile mouse

model for Bloom syndrome. Moreover, *Blm*$^{m3/m3}$ mice have been reported to have predispositions to cancer, such as leukemia and lymphoma. Therefore, *Blm*$^{m3/m3}$ mice might be an ideal mouse model for preclinical trials. However, the characteristics of HSCs in terms of genomic instability, such as SCE elevation and MMC hypersensitivity, remain unclear. In this study, we analyzed the frequency of SCE and sensitivity to MMC in HSCs from *Blm*$^{m3/m3}$ mice to identify markers that can potentially measure gene therapy effects. Methods: 1. MMC sensitivity assay: Lentiviral vectors (LV) encoding the *BLM* gene with green fluorescent protein (GFP) were pseudotyped using the vesicular stomatitis virus (VSV-G) envelope (VSV-G-BLM-GFP-LV). Harvested HSCs from *Blm*$^{m3/m3}$ mice and wild-type (WT) C57BL/6 mice were pre-stimulated in stem cell growth media, supplemented with murine cytokine thrombopoietin (100 ng/mL), Flt3L (100 ng/mL), and stem cell factor (100 ng/mL) for 5 h at 100,000 cells/mL. Pre-stimulated HSCs were cultured for 48 h in medium containing only hexadimethrine bromide at a final concentration of 4 μg/mL and in medium containing hexadimethrine bromide along with VSV-G-BLM-GFP-LVs (MOI: 50) at 100,000 cells/mL. For methylcellulose colony-forming unit assays, 500 cells of transduced HSCs were plated into MethoCult M3534 medium. To elucidate the sensitivity of HSCs to MMC, they were cultured in MethoCult medium containing increasing concentrations of MMC (0-0.5 nM MMC). After 14 days of culturing, the number of colonies was counted. 2. SCE analysis: HSCs collected from *Blm*$^{m3/m3}$ and WT mice were incubated with bromodeoxyuridine (BrdU). The number of SCEs in metaphase chromosome spreads was then counted in 20 HSCs from each of the *Blm*$^{m3/m3}$ and WT mice. Results: 1. MMC sensitivity assay: *Blm*$^{m3/m3}$ HSCs did not show higher sensitivity to MMC than WT HSCs. VSV-G-BLM-GFP-LV transduction did not improve the sensitivity of MMC in *Blm*$^{m3/m3}$ HSCs. 2. SCE analysis: The number of SCEs in *Blm*$^{m3/m3}$ HSCs was approximately two-fold higher than that in WT HSCs. Discussion: According to our experiments, *Blm*$^{m3/m3}$ HSCs did not show high sensitivity to MMC. One of the reasons might be that the Blm mutant protein in *Blm*$^{m3/m3}$ mice has been reported to retain 25% WT activity. Therefore, this retention of WT activity of the Blm mutant protein may result in a lack of high sensitivity to MMC. However, *Blm*$^{m3/m3}$ HSCs showed a two-fold increase in the number of SCEs compared with WT HSCs. In conclusion, to advance pre-clinical trials, the evaluation of SCEs can be used as a valid marker to measure the functional effect of gene transfer in HSCs of *Blm*$^{m3/m3}$ mice.

1650 Transient Immune-Modulation Can Reduce the Immune Response Induced After Systemic *In Vivo* Administration of INS-101 Lentiviral Gene Therapy

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Innoskel is developing a lentiviral-based gene therapy approach to treat individuals with Spondyloepiphyseal Dysplasia congenita (SEDc). SEDc is a rare genetic bone disorder caused by mutations in the *COL2A1* gene and is associated with progressive skeletal deformities that are life-threatening and require repeated surgical procedures. Our gene therapy product INS-101 is a lentivirus (LV) that delivers a wild-type *COL2A1* transgene to cartilage growth plates to correct the molecular cause of the disease. Preclinical evaluation of INS-101 has demonstrated efficacy in a SEDc mouse model. INS-101 systemic injection *in vivo* is associated with an immune response to the LV. This response might decrease therapeutic efficacy, lead to adverse events, and prevent redosing of INS-101 in patients. We characterized the LV-induced immune response in mice, primates or minipigs and evaluated the ability of immune-modulatory drugs to reduce or prevent it. IgG and IgM responses directed to the LV, as well as neutralizing antibodies were detected in the serum of LV-injected animals. The release of pro-inflammatory cytokines was also observed shortly after injection, and a specific LV-directed cellular response was seen by ELISpot using isolated splenocytes. We tested several immunosuppressive drugs via repeated injections around the time of the LV administration in mice: effects of Anti-CD20, Anti-BAFF, Methotrexate (MTX), Mycophenolate Mofetil (MMF), Tacrolimus, Prednisolone and Rapamycin were monitored alone or in combination. LV-specific IgG and IgM levels, as well as neutralizing antibody titers, were significantly reduced by some individual or combination treatments. The ability of a regimen including Prednisolone, Tacrolimus and MMF in reducing the LV-induced humoral immune response was also demonstrated in minipigs. Together, our results indicate that a transient immune-modulatory treatment can effectively and durably impair the LV-induced immune response.

1651 Immunosafety of AAV Gene Therapy Vectors: Induction of Cytokine Release and Complement Activation

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Gene therapy mediated by recombinant adeno-associated virus (rAAV) is one of the most promising approaches for the treatment of a variety of inherited and acquired diseases. Multiple variants of AAV vectors have established themselves as powerful tools for *in vivo* gene transfer, allowing long lasting and safe transgene expression in a variety of clinical trials. Nevertheless, the immune response against these vectors, and particularly its capsid, remains a major limitation for gene transfer. Upon administration, rAAV vectors stimulate both innate and adaptive immune responses. The innate response stimulates adaptive immunity, leading to the formation of anti-AAV capsid antibodies, which prevent the transduction of target cells and preclude successful vector re-administration. Moreover, the innate immune response can contribute to toxicities. Adverse events in several systemic gene therapy clinical trials have been linked to complement activation, leading to decreases in platelets and red blood cells, and to acute kidney injury. We assessed

the effects of AAV2 and AAV8 vectors on the production of a large panel of cytokines and chemokines in whole blood from healthy human donors (n=18). In order to investigate the role of pre-existing anti-AAV antibodies on cytokine release and complement activation, these experiments were conducted in whole blood from both seropositive and seronegative donors. The results showed that AAV vectors could trigger low but detectable levels of cytokines and chemokines (IFN-α, IFN-β, IFN-γ, IL-1b, IL-2, IL-6, IL-8, IL-10, IP-10, MCP-1, MIP-1α, MIP-1β and TNF-α) in whole blood from a proportion of the AAV-seropositive donors tested, but not in all of them. Notably, the IFN-α release was almost undetectable with empty AAV capsids, supporting the hypothesis that IFN-α release is triggered by the genome through TLR9 activation. We used single-cell RNA sequencing to characterize the cytokine-producing cells in whole blood at different time points. The results showed that the production of cytokines was mainly driven by monocytes, NK cells and T cells. Interestingly, increases in several of these cytokines and chemokines were observed in the serum of mice after injection of a high AAV8 dose. We also investigated the mechanism of complement activation by AAV capsids by measuring increases in C3a and C5a in whole blood from a large cohort of donors (n=20). Complement activation occurred in an AAV dose-dependent manner and mainly in the presence of anti-AAV antibodies. No difference was observed in complement activation whether empty or full AAV capsids were used for whole blood treatment. Our results show that complement activation by AAV is antibody-dependent and mediated through the classical pathway. Our work also highlights the interest of monitoring cytokine release in clinical trials to better understand its impact on the adaptive immune response and the clinical outcome. Lastly, investigating the effects of AAV vectors on cytokine release in whole blood may allow identifying less inflammatory and safer AAV vectors.

1652 A Recombinant Adenovirus Vector Containing the *synNotch Receptor* Gene Inhibits Tumor Growth of Triple-Negative Breast Cancer in a Mouse Xenograft Model

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Introduction: Triple-negative breast cancer (TNBC) is one of the most difficult molecular subtypes of breast cancer to treat. We developed a recombinant replication-deficient adenoviral vector (Ad-CD44-N-HIF-3α4) containing a gene encoding a synthetic Notch (synNotch) receptor composed of the extracellular domain of CD44 (CD44-ECD) and the hypoxia-inducible factor (HIF)-3α4 connected by the Notch core regulatory region. CD44 is a cell surface adhesion receptor and a known cancer stem cell marker in breast cancer and other malignancies. HIF-3α4 is a dominant-negative regulator of HIF-1α in TNBC cells. The CD44-ECD in the synNotch receptor acts as a CD44 decoy receptor, and after a ligand binds to the CD44-ECD, HIF-3α4 is released, resulting in the inhibition of both CD44 and HIF-1α signaling pathway in cancer cells by transduction of *CD44-Notch-HIF-3α4* fusion gene into TNBC cells. **Methods:** In this study, we employed a human TNBC cell line, MDA-MB-231. Gene transduction with recombinant adenoviral vector containing a fusion gene *CD44-Notch-HIF-3α4* (Ad-CD44-N-HIF-3α4) and the gene expressions were determined. MDA-MB-231 cells were cultured under conditions of hypoxia (≤2% O_2). Ad-CD44-N-HIF-3α4 and a control Ad-LacZ were infected into cells at 50 multiplicities of infection (MOIs), respectively. To determine the cell proliferation, 2,000 cells/well MDA-MB-231 cells were treated with 50 MOI of Ad-CD44-N-HIF-3α4 or Ad-LacZ and incubated at 37°C for 5 days at hypoxic or normoxic condition *in vitro*. *In vivo* study, MDA-MB-231 cells were subcutaneously inoculated into mice. Intratumoral injections were performed with 1×10^9 PFU of adenoviral vectors Ad-CD44-N-HIF-3α4, Ad-LacZ, and PBS control. **Results:** Besides the endogenous CD44 protein, we confirmed the transductions of decoy CD44-ECD proteins in MDA-MB-231 cells in dose dependent manners. The mRNA expression of *HAS2* in MDA-MB-231 cells was significantly increased compared to SV-HUC-1 cells, while the mRNA expression of *HAS1* and *HAS3* were not changed ($p<0.01$). In addition, the relative gene expressions of *survivin* and *CCL2*, downstream genes of CD44, in the cells infected with Ad-CD44-N-HIF-3α4 were significantly lower than in cells infected with Ad-LacZ and control cells under culture conditions of hypoxia ($p<0.01$). The relative gene expression of *vascular endothelial growth factor* (*VEGF*), a hypoxia target gene, was significantly decreased in the cells infected with Ad-CD44-N-HIF-3α4 than that in control cells under culture conditions of hypoxia ($p<0.01$). In the cell proliferation analysis, Ad-CD44-N-HIF-3α4 significantly inhibited the cell growth of MDA-MB-231 cells in normoxia and hypoxia conditions compared to cell only (no treatment) group ($p<0.01$). Transwell invasion assay, Ad-CD44-N-HIF-3α4 significantly inhibited the cell migration of MDA-MB-231 cells in hypoxia condition but not in normoxia condition ($p<0.05$). Furthermore, Ad-CD44-N-HIF-3α4 significantly suppressed tumor growth compared to Ad-LacZ or PBS control in mice ($p<0.01$). The expression of CD44 was remarkably increased in mice tumors with the treatment of Ad-CD44-N-HIF-3α4. **Conclusion:** Ad-CD44-N-HIF-3α4 inhibited the both signaling pathways of CD44 and HIF-1α in MDA-MB-231 cells under conditions of hypoxia *in vitro*, and significantly suppressed tumor growth both *in vitro* and *in vivo*. These findings indicated that Ad-CD44-N-HIF-3α4 has a high clinical applicability for invasive types of TNBC.

1653 Transcriptomic Profiling of the Mouse Hippocampus After Intracerebral Injection of Cas9 Nanocapsule Genome Editors

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Evaluating the editing efficiency and cell-type specificity of genome editors is a critical task for developing somatic cell genome editing strategies, especially for those that target the brain. Standard methods rely on deep sequencing at the on-target site combined with immunohistochemistry within treated animal model systems to enumerate the types of edited cells in select tissues after administering genome editors. Animal reporter systems that express fluorescent

proteins after successful on-target genomic editing provide robust platforms to evaluate the number of edited cells but typically incorporate limited opportunities to co-register cell-type markers with the reporter protein. Thus, molecular characterization of an edited cell typically is limited to 2-4 cell-type markers that can be imaged simultaneously with the fluorescent reporter. Here, we report on a new approach to deeply characterize the transcriptome of edited cells within the brain of Ai14-tdTomato reporter mice. We performed single nucleus RNA sequencing (snRNA-seq) on nuclei isolated from hippocampi after intracranial injection of nanoparticle genome editors into the dorsal hippocampus. We utilized a previously developed biodegradable nanocage (NC) capable of delivering a preassembled SpyCas9 protein-gRNA ribonucleoprotein complex (RNP). RNPs targeting the Ai14 loxP-STOP cassette were encapsulated into these NCs and delivered into the dorsal hippocampus via intracranial injection. After performing snRNA-seq, we observe the capture of ~500-15000 unique transcripts per nuclei and robust Ai14-tdTomato reporter expression in nuclei from neuronal, glial, and oligodendrocytic cells. Additionally, differential cell-type specific transcriptomic shifts were identified primarily in immune and cell signaling pathways between treatment with recombinant adeno-associated viral vectors (rAAV) or NC. Overall, transcriptional profiling provides a high-resolution and complementary method to examine the cellular outcomes from genome editing within animal reporter systems, which has high potential to inform the clinical development of genome editing therapeutics.

1654 Bioprinted Liver Tissues as a Cell Therapy for Phenylketonuria

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Background: Phenylketonuria (PKU) is an autosomal recessive disorder, affecting approximately 1:10,000 newborns worldwide. PKU is caused by mutations to the *PAH* gene, resulting in aberrations in the phenylalanine hydroxylase (PAH) enzyme. Given that PAH converts phenylalanine to tyrosine, individuals with PKU have elevated plasma phenylalanine concentrations, resulting in profound intellectual disability if left untreated. Current treatment for PKU involves controlling plasma phenylalanine levels via a phenylalanine-restrictive diet. Adherence to this diet is problematic, with compliance decreasing in adolescence and adulthood. While efficacious, liver transplantation is not a viable treatment for PKU due to its invasiveness, long-term immune suppression, and limited supply of donor organs. Herein, we use a unique microfluidic 3D bioprinting technology to demonstrate the ability of a bioprinted hepatocyte-containing tissue to metabolize phenylalanine to treat PKU. **Methods:** Primary human hepatocytes (PHHs) were aggregated into spheroids with and without mesenchymal stromal cells (MSCs). Spheroids were then 3D bioprinted in an alginate-based tissue using Aspect Biosystems' microfluidic 3D bioprinting technology. To assess phenylalanine metabolism *in vitro*, bioprinted liver tissues were challenged with media containing 2 mM phenylalanine. Conditioned media was collected over 7 days and phenylalanine was quantified using a fluorogenic assay. Additionally, bioprinted liver tissues were assessed for viability, albumin secretion, gene expression, and Cyp3A4 activity. **Results:** Bioprinted tissues containing PHHs and PHHs/MSCs were able to metabolize >500 nmol of phenylalanine/million PHHs/day, when challenged with PKU-relevant concentrations of phenylalanine *in vitro*. Gene expression analysis indicates bioprinted tissues express PAH, suggesting their ability to restore PAH activity. Furthermore, these bioprinted tissues demonstrate additional liver specific function, such as albumin secretion (>10 µg/million PHHs/day) and cytochrome P450 activity over a period of 7 days. **Conclusions:** Bioprinted hepatocyte-containing tissues are capable of metabolizing physiologically relevant levels of phenylalanine and maintaining function *in vitro* in the long term. This demonstrates that bioprinted tissues containing PHHs represents a viable therapeutic strategy for the treatment of PKU. Further studies are warranted to assess the efficacy of bioprinted hepatocyte-containing tissues on phenylalanine metabolism in a small animal model of PKU.

1655 Thermosensitive Hydrogel-Based Spatiotemporally Controlled Gene Therapy

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Biomaterials such as hydrogel, microneedle and nanofiber are widely used in drug and gene delivery. Especially, hydrogels are unique biomaterials that have demonstrated tremendous potential in localized drug delivery with high drug-loading ability, great biocompatibility, and sustained & controlled drug release. In this study, we aimed to develop a dual delivery system of thermosensitive hydrogel incorporating DNA-complexes with various functional polymers and cell penetrating peptides for spatiotemporally controlled gene therapy. This approach involves developing thermosensitive mPEG-PLGA hydrogel possessing sol-gel transition property in the response to temperature change and facilitating the encapsulation and sustained release of therapeutic genes. For an enhanced gene delivery to target cells, we formulated a DNA-complex with various polymers and cell penetrating peptides, which could enhance cell uptake, endosomal escape, and transfection efficiency. Indeed, DNA complexed with RALA peptide showed great gene delivery efficiency with lower cytotoxicity compared with commonly used transfection agents. Overall, this dual delivery system demonstrates excellent capability for spatiotemporally controlled gene therapy, thereby exhibiting great potential for use as a gene therapeutic platform for many diseases.

1656 Safety of Ascending Doses of AAVrh.10hFXN to Treat the Cardiac Manifestations of Friedreich's Ataxia

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Friedreich's ataxia (FA) is caused by a GAA trinucleotide expansion in the first intron of the frataxin gene (FXN), resulting in a reduction in frataxin mRNA and protein levels. Following development of neurologic disease, individuals homozygous for FA develop cardiac dysfunction at age 20 to 25 and cardiac disease is the leading cause of death at an average age of 37.5 yr. Previous studies in murine models the cardiac manifestations of FA established that adeno-associated virus (AAV) serotype rh.10 expressing human frataxin (AAVrh.10hFXN) at an intravenous dose of $1.8x10^{12}$ gc/kg mediated cardiac frataxin levels in the range of asymptomatic heterozygous carriers, resulting in improvement in the cardiac manifestations of FA (Munoz Zuluaga C, et al, Mol Ther 2022; 30 4S1:444). This study sought to establish the safety of this dose in mice and nonhuman primates for potential use in a human clinical trial. AAVrh.10hFXN was administered intravenously (IV) to wildtype C57Bl/6N mice (n=24F/24M per dose cohort) at 3 increasing half-log doses of $1.8x10^{12}$ ("low"), $5.7x10^{12}$ ("mid"), and $1.8x10^{13}$ gc/kg ("high"), with a control group administered PBS. Mice were weighed and evaluated for health and behavioral assessments weekly. Mice were evaluated at 1, 3 and 10 months for toxicity, FXN levels in liver and heart, troponin levels, total antibody and neutralizing antibody titers, biodistribution of the AAV genome and organ histopathology. At the low dose of $1.8x10^{12}$ gc/kg there were no adverse treatment-related findings except 1 male mouse in the 10-month necropsy group had hepatocellular carcinoma. The mid-dose cohort showed mild to moderate hepatic toxicity evident in clinical chemistry and in histopathology at 3 months, and 3 of 12 male mice had hepatocarcinoma at 10 months. High dose mice expressed greatly elevated frataxin levels in liver, had decreased body weight and severe hepatic toxicity. In the context that hepatocarcinoma is a sporadic observation in inbred mice treated with AAVs but has not been observed in nonhuman primates or humans, to provide additional confidence in the safety profile of AAVrh.10hFXN, we evaluated the safety of AAVrh.10hFXN administered IV in African Green nonhuman primates (NHP; n=10), at $5.7x10^{11}$ or $1.8x10^{12}$ gc/kg doses (n=2/sex/dose), plus PBS controls (1/sex). All NHPs were administered prednisolone immunosuppression pre-dose and continuing throughout the study. All animals survived to the scheduled euthanasia, 3 months post-dose. No abnormal clinical signs were observed and there were no vector-related effects on body weight, organ weight, macroscopic differences or blood parameters. There were no microscopic findings of hepatocarcinoma or hepatotoxicity. Echocardiograms 3-months post-vector administration were normal. On average, the increase in cardiac FXN expression after AAVrh.10hFXN administration with $5.7x10^{11}$ and $1.8x10^{12}$ gc/kg doses was 6.5 and 37%, respectively, over the PBS-treated controls. Together, these data identify the safe doses of AAVrh.10hFXN relevant for the treatment of the cardiac manifestations of FA.

1657 Full-Length rAAV Sequencing for Mixture Population Characterization Using Highly Accurate Long Reads

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Recombinant Adeno-associated virus (rAAV) is one of the most actively investigated gene therapy vehicles. Understanding the quality of rAAV vectors by assessing the presence of cellular impurities such as truncations, chimeras, and host genome integrations are all essential for ensuring the purity and safety of the vector. While next-generation sequencing has been applied for assessing the quality of rAAV vectors, short read length technologies cannot sequence the full-length AAV molecules, which are necessary for identifying these impurities. Furthermore, short read sequencing cannot accurately distinguish or quantify mixed populations that might contain both scAAV and ssAAV. We demonstrate that using highly accurate long reads (HiFi reads) from PacBio, we can accurately characterize rAAV mixtures. PacBio SMRT sequencing is a long-read sequencing technology that can produce reads of >Q20 (>99%) for insert sizes of 10 kb - 20 kb and longer, which easily cover the entirety of typical rAAV constructs. To demonstrate the PacBio long read sequencing can accurately characterize AAV constructs, we purchased scAAV and ssAAV constructs from commercial vendors. We created sequencing libraries from both supposedly pure (scAAV or ssAAV-only) vectors and also an artificially mixed (scAAV with ssAAV) population. The long read data was able to characterize the proportion of vector genomes for truncation (full-length or partial), purity, structure (self-complementary or single-strand). To summarize, we show that the long read sequencing has the ability to accurately and comprehensively assess the safety, potency, and purity of rAAV vectors, making it a valuable tool for the quality assessment of rAAV genome populations.

1658 An Illustrative Example from the PaVe-GT Program for Navigating FDA Orphan Drug and Rare Pediatric Disease Designation Applications

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There are over 10,000 known rare diseases and more than 90% have no cure or treatment. Many of these diseases occur in very small patient populations, leading to a lack of commercial interest in developing new treatments for such orphan indications. Platform Gene Vector Therapy (PaVe-GT) is developing gene therapies for four disorders with very low prevalence. Along the process of PaVe-GT

implementation, we plan to publicly share our regulatory and scientific experience from different stages utilizing illustrative examples. The first gene therapy candidate of PaVe-GT, adeno-associated virus, AAV9-hPCCA to treat PA has been granted Orphan Drug Designation (ODD) and Rare Pediatric Disease Designation (RPDD) from the FDA. ODD and RPDD are FDA programs to facilitate the development of orphan drugs in the US by providing financial incentives for sponsors. Our application process is outlined in accordance with FDA requirements. Described in detail are: a) Prerequisites for an ODD and RPDD application and a checklist of the requirements, as defined by the FDA; b) Approaches to providing proof of direct benefit to trial participants and potential therapeutic effectiveness in the rare disease; c) Steps and approaches to accurately estimate patient population size in the US, required to establish the orphan status of the disease or condition; d) Resources for preparing a successful ODD/ RPDD application; e) Templates and AAV9-hPCCA redacted applications. Both these programs for orphan drugs have been shown to have a positive correlation with innovation, investor valuation and investments in rare disease research and development. The resources and process presented to prepare an ODD application can benefit parties with limited experience and understanding of the regulatory requirements and serve as a guide in the preparation of a similar application for their orphan product.

1659 Moving Past the BBB - Screening for the Most Functional CNS-Tropic AAV

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The use of recombinant adeno-associated viral vectors (rAAV) as a gene delivery tool has demonstrated great potential for both therapeutic gene therapy and scientific research. One of the key benefits of rAAV is the ability to alter the tropism and transduction efficiency of different serotypes and variants by engineering the viral capsid. Recent advances in vector bioengineering technologies have improved our understanding of vector interactions with target cells, allowing researchers to rapidly evolve novel AAV variants and introduce targeted changes within existing rAAV capsids. These changes aim to improve overall vector properties, such as tropism, production capacity, stability, and immunogenicity. Due to the increasing maturity of this technology, there are now hundreds of academic and commercial teams working in a wide array of preclinical models to engineer AAVs for a range of organs and cell types. However, this decentralised approach to vector bioengineering leads to a lack of studies that directly compare vectors developed by individual groups. Vectors developed for specific targets are also not evaluated for their potential superior properties in other applications. Given the vast number of engineered rAAVs now available, it is worth considering whether efficient vectors can be repurposed for targeting other organs of interest, such as the central nervous system (CNS). Despite the progress in rAAV targeting, the CNS remains a challenging target due to the limited ability of AAV serotypes to efficiently cross the blood-brain-barrier (BBB) and transduce the CNS when injected intravenously (IV). Intravenous injections can be disadvantageous for targeting the CNS as they require vectors to be capable of crossing the highly selective BBB, whilst they also increase the risk of off-targeting peripheral organs, such as the liver. Additionally, there is a lack of rAAVs that can target specific regions/cell types in the CNS. We have developed an AAV screening tool to assess the ability of known and characterised AAV variants to transduce the CNS of mice when injected passed the BBB via intrathecal (IT) or intracerebroventricular (ICV) injection. Fifty-seven (n=57) AAV variants were screened head-to-head to identify top-performing candidates at the RNA level within specific brain regions, including cerebellum, hippocampus, cortex, and spinal cord of both adult and P0 mice. Interestingly, top-performing vectors were similar in both injection methods, however, results demonstrate unique vector tropisms for specific regions and cell types in the CNS. Following the screening process, top AAV variants were assessed individually to evaluate and compare transduction efficiency and tropism. Following this exciting proof-of-concept study, we currently seek to expand into more clinically relevant models, such as non-human primates (NHP).

1660 GMP Production of a Cas9 3xNLS Protein for the Treatment of Sickle Cell Disease

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Sickle cell disease is a genetic red blood cell disorder that causes hemolysis, anemia, vaso-occlusion, organ damage, and pain in millions of individuals across the world. Gene editing using a modified Cas9 protein and guide RNA to reactivate fetal hemoglobin offers a potentially curative therapy for this disease. In this work, we describe the growth and purification of a tag-less modified Cas9 3xNLS protein from *E. Coli* in a GMP suitable process for use in St. Jude's gene editing program to treat sickle cell disease, termed St. Jude Autologous Genome Edited Stem cells (SAGES-1). SAGES-1 is an autologous gene editing strategy to target the -113 site of the γ-globin promoter to induce HbF. While affinity tag purification of recombinant proteins is suitable for research purposes, this approach is not preferred in Good Manufacturing Practice (GMP) applications due to the need to remove and test for contaminating affinity labels in the final product. A bioreactor (5L) is inoculated from an overnight shake flask culture of a producer cell line. Cells grow overnight in the bioreactor at 30°C with an exponential feed until they reach an OD_{600} of 60-80, after which the temperature is decreased to 20°C and the culture is induced with IPTG. Culture is harvested 21-23 hours after induction. Harvested culture is homogenized and filtered before purification over a Sepharose SP column, incubation with allantoin, and further purification over a Ceramic Hydroxyapatite Type 1 column. The purified Cas9 protein is diafiltrated and concentrated to 12 mg/mL by tangential flow filtration before vialing. *E. Coli* produces endotoxin, which can stimulate an immune response of fever and inflammation. Our initial purification process left with a high level of endotoxin in the final product. Addition of 100 mg/mL allantoin, a small crystalline powder, and the use of a Ceramic Hydroxyapatite Type 1 column reduced this level to acceptable levels. The average yield of the process over three runs was

1.2 mg protein per g of wet pellet weight and the purity was greater than 95% as measured by reverse phase UPLC, SDS-CGE, and Size Exclusion Chromatography. Furthermore, we have compared Cas9 3xNLS protein made with this method over several batches and shown the batches to be consistently highly active in both *in-vitro* cleavage assays and in CD34+ hematopoietic stem cells. These batches of GMP-like Cas9 are going into our IND-enabling *in vivo* pharmacology and toxicology studies.

1662 Microfluidic Production of Nanogels as Alternative Triple Transfection Reagents for the Manufacture of AAVs

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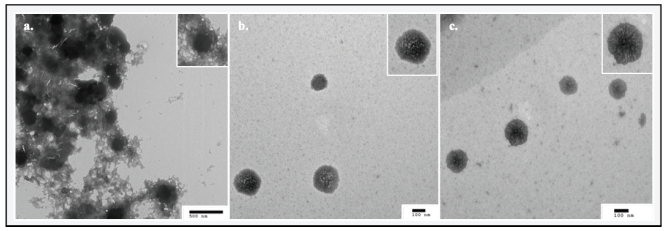
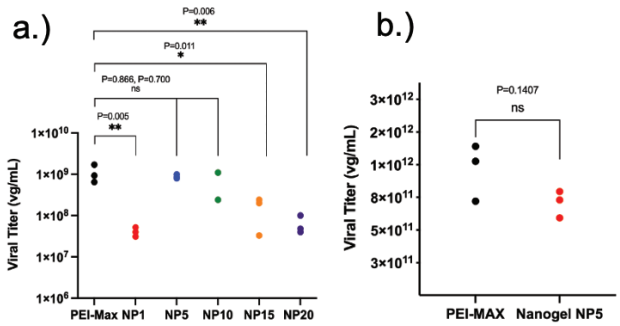
Introduction- AAVs represent one of the most widely studied gene delivery systems. Their production, however, remains challenging in terms of yield, cost-effectiveness and large-scale manufacture. In this work, we present an alternative triple transfection method for AAV9 production, using nanogels produced by microfluidics, compared to polyethyleneimine-MAX (PEI-MAX) polyplexes. We anticipate that this will facilitate AAV manufacturing in becoming a continuous process with high yields, rapid production and reduced costs. **Materials and Methods - Nanogel production by microfluidics** -The microfluidic coaxial flow reactor (CFR) is adapted from Whiteley *et al1*. Nanogels contained pDNA at weight ratios of 1:1:2 (pAAv: pDG9: pHGTI) at nitrogen: phosphate (NP) ratios of 1 to 20. **Small and large-scale AAV production** - PEI-MAX (2mg/mL) was added to pDNA (1:1:2 weight ratio). HEK293T cells were plated in 6-well plates and nanogels and PEI-MAX was added at 2.5µg pDNA/well. The AAV in the supernatant was measured after 72h. For large-scale production, a total pDNA quantity of 52.5µg/dish of PEI-MAX and nanogel formulations was added to 150cm^2 dishes. After 72 hours, the cells and supernatant were collected, concentrated and purified. **Results and Discussion - Particle characterization**- The aim was to produce nanogels below 200 nm and PDI below 0.3. The nanogels ranged from 116.3 nm ± 10.8 at NP10 to 187.0 nm ± 4.7 at NP5, with NP10 and 20 demonstrating significantly smaller sizes than the PEI-MAX. PDI values below 0.3 indicate a high degree of monodispersity[2]. PEI-MAX demonstrated a PDI of 0.321 ± 0.005, significantly larger than NP5 at 0.199 ± 0.013 and NP20 at 0.154 ± 0.018. TEM images of PEI-MAX polyplexes, NP10 and NP20 nanogels (Figure 1 (a-c) respectively) show the PEI-MAX polyplexes formed aggregates revealing instability.**AAV production** - The nanogels were transfected in HEK293T cells in 6-well plates. The nanogels producing the highest AAV titre were selected for large-scale manufacturing. Figure 2a shows that NP5 and NP10 nanogels produce AAV at 8.83x10^8 vg/mL ± 9.71x10^7 and 8.13x10^8 vg/mL ± 4.97x10^8, statistically equivalent to PEI-MAX at 1.09x10^9 vg/mL ± 5.44x10^8. Decreases in NP ratio to 1, resulted in a significant reduction to 4.10x10^7 vg/mL ± 1.05x10^7. The production of AAV from NP5 nanogels (7.41x10^{11} vg/mL ± 1.36x10^{11}) at large scale (Figure 2b) showed no significant difference to the titer from PEI-MAX polyplexes (1.24x10^{12} vg/mL ± 4.4x10^{11}). **Conclusions** - Microfluidic CFRs produce nanogels with viral titers equivalent to PEI-MAX. Nanogels are stable and show greater cost-effectiveness than PEI-MAX, whilst microfluidics allows for larger-scale continuous production. **References** - (1) Z. Whiteley, H. M. K. Ho, Y. X. Gan, L. Panariello, G. Gkogkos, A. Gavriilidis and D. Q. M. Craig, *Nanoscale Adv.*, 2021, **3**, 2039-2055. (2) L. Xu, Y. L. Wang, Xing, G. Yang, R. J. Falconer and Chun-Xia Zhao, *Adv. NanoBiomed Res.*, 2021, **2100109**, 51-79.





1663 Development of a Cas13-Based RNA Targeting Therapy for Neovascular Age-Related Macular Degeneration (nAMD) and Diabetic Macular Edema (DME)

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Background: Age-related macular degeneration (AMD) is a major cause of visual dysfunction for adults over age 50 of which the neovascular AMD (nAMD) accounted for 90% blindness in AMD. The vascular endothelial growth factor (VEGF), an angiogenic growth factor, plays a crucial role in nAMD and diabetic macular edema (DME). Although anti-VEGF agents are effective and safe in treating nAMD and DME, long-term efficacy decreases over time due to the need for repeated injections impacting patient compliance with treatment regimens, patients still may lose vision during the 7th or 8th year of the treatment. These frequent intravitreal injections can increase the risk of complications, including submacular hemorrhage, intraocular hypertension, endophthalmitis, and retinal detachment. Furthermore, many patients with nAMD do not respond well to the standard anti-VEGF therapy. **Methods**: We developed a Cas13-based RNA targeting therapy, HG202, that used a unique strategy of AAV vector combined with high-fidelity CRISPR/Cas13 RNA targeting technology to knock down the expression of VEGFA in the local retina. The choroidal neovascularization (CNV) was induced in mouse eyes

by laser photocoagulation. Fundus fluorescein angiography (FFA) and optical coherence tomography (OCT) were conducted 7 days after injury. Afterwards, choroidal flatmounts were immunostained for CNV detection. **Results**: Significant editing efficiency was observed in both *in vitro* and *in vivo*. More than 40% *Vegfa* mRNA expression was suppressed by subretinal delivery of HG202 at the optimal dose in retina (n=8) of mice. The area of laser-induced CNV was markedly reduced in mice eyes treated with HG202 at the optimal dose (87% reduction, p<0.0001, n=14). By contrast, the inhibition efficiency of AAV-anti VEGF was lower than that of HG202 (70% reduction compared to the vehicle control, p<0.01, n=14) and Aflibercept was only half as much as that of HG202 (47% reduction, p<0.01, n=18) (Figure 1). With a single injection, we achieved a therapeutic effect of at least 13 weeks in the mouse model. Additionally, HG202 was well tolerated in mice at a certain dose range. **Conclusions**: Compared with DNA editing or protein inhibitors strategies, we blocked VEGFA signaling by Cas13-mediated mRNA knockdown, enabling high efficacy, and theoretically avoiding permanently changes of DNA sequence. In summary, a single subretinal injection of HG202 enabled long-term transgene expression and RNA editing, avoiding 1- or 2-month repeated injections, and providing long-lasting therapeutic benefits for patients (with or without anti-VEGF antibody) to treat nAMD and DME. To the best of our knowledge, we are the only group using Cas13-based RNA target strategy to treat nAMD patients with or without anti-VEGF antibody.

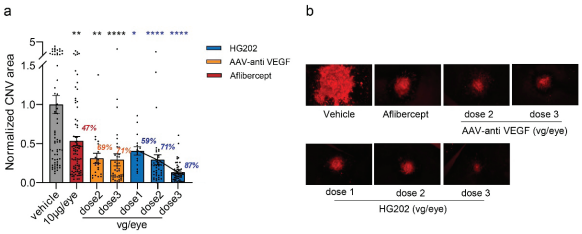

Figure 1. Inhibition of laser-induced CNV development by H202 treatment

1664 Circular RNAs Localize to Synapses in the Human Brain and Differentially Expressed in Alzheimer's Disease Implicating Critical Roles for Synaptic Function Which Could Present New Targets for Gene Therapy

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Circular RNAs (circRNAs) are unique transcript isoforms that may play critical regulatory roles in the human brain which could have impacts in the progression of neurodegenerative disorders such as Alzheimer's Disease (AD). CircRNAs are produced by back-splicing a later exon to a preceding exon creating a closed-loop structure. They can perform a variety of functions, in addition to being translated, including acting as microRNA "sponges" and sequestering RNA binding proteins. CircRNAs also persist in the cytoplasm due to exonuclease resistance. These multifunctional roles distinguish them as attractive targets for gene therapy. Alzheimer's Disease (AD) is the most prevalent form of dementia and is characterized by progressive accumulation of plaques consisting of amyloid beta peptide and neurofibrillary tangles of hyperphosphorylated tau protein. These molecular pathologies are followed by synaptic and neuron loss as well as cognitive impairment. Previous research demonstrated circRNAs preferentially localize to synapses and expression of many are perturbed in AD. We corroborate this evidence with the addition of novel insights using new techniques and statistical approaches, and we follow this up with determining functional roles for identified circRNAs. To discover and quantify RNA transcripts localized to the synapse in AD, we acquired more than 30 postmortem human brain samples including AD patients and controls. From homogenized brain tissue, we fractionated synaptic particles (synaptosomes) using sucrose gradient ultracentrifugation. Next, we extracted RNA from both the synaptosome fraction and homogenate, then acquired RNA sequencing data using a ribo-removal total RNA strategy which captures non-canonical isoforms. By comparing synaptosome to homogenate, we confirmed that the overwhelming majority of circRNAs are enriched in synapses. Furthermore, by comparing synaptosomes between AD and control, we discovered that there are also many circRNAs that differentially localize to synapses in AD indicating potential targets for gene therapeutic intervention in an attempt ameliorate disease. Among the most significantly differentially localized circRNAs, two isoforms of the gene *GSK3B* stood out. One isoform is a circle of exons 7, 8, and 9, and the other isoform is a circle of exons 9 and 10. Most interesting is that the former isoform is upregulated in AD with a $\log_2$ fold change of 1.5 whilst the later is downregulated in AD with a $\log_2$ fold change of -1.6. The discovery that there are two unique circular isoforms of *GSK3B* each differentially localized at synapses in AD is noteworthy since GSK3B has an established role in tau hyperphosphorylation. To investigate the impact of each of these isoforms, we transfected SH-Sy5y cells with the circ*GSK3B* exons in a plasmid containing complementary Alu elements that facilitate their circularization. We find that transfection of each of these plasmids causes contrasting protein abundance differences in the GSK3B protein as well as its phosphorylation state which could demonstrate a connection with AD molecular pathologies. We hypothesize that these circRNAs have further downstream impacts on protein expression including tau. This provides a basis for which protein expression could be modulated to treat disease by the delivery or manipulation of circRNAs which is an exciting new prospect given their multifunctional roles and stability in the cytoplasm. Here we present our work to uncover the significance of these findings which represents a novel investigation into this class of understudied molecules.

1665 PN Modification of Stereopure GalNAc-siRNAs Enhances Durability of Human *HSD17B13* Silencing in Transgenic Mouse Model

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We aimed to develop siRNAs to suppress expression of the clinically relevant liver gene *Hydroxysteroid 17-beta dehydrogenase 13* (*HSD17B13*). A loss of function genetic variant of *HSD17B13* has been associated with a reduced risk of chronic liver disease and progression from steatosis to steatohepatitis, suggesting that decreasing *HSD17B13* expression in liver could be protective against liver disease. To develop *N*-Acetylgalactosamine (GalNAc)-conjugated siRNAs targeting hepatic *HSD17B13*, we first interrogated the impact of chemical and stereochemical modifications to sense and antisense strands containing chiral phosphorothioate (PS) and phosphoryl guanidine (PN) modifications using mouse *Transthyretin* (*Ttr*) as a benchmark. We evaluated hundreds of stereopure GalNAc-siRNAs—those in which the chiral configuration (*R*p or *S*p) of chemically modified backbone linkages (PS or PN) are controlled at each position—in comparison with stereorandom GalNAc-siRNAs designed based on formats previously reported to have enhanced stability. In primary mouse hepatocytes and mouse liver, we show that a 5′-*R*p*S*p PS in the antisense strand is favored in a format with high 2′-deoxyfluoro (2′-F) content and without PN linkages. With the addition of PN linkages, the configuration of the 5′-end PS linkages becomes less important. We also show that an *S*p PS configuration on both ends of the sense strand is favored. The impact of the PN backbone on activity depends on its position in the backbone, the chirality of the linkage, and nearby 2′-ribose modifications. The potency and durability benefits observed *in vivo* with these stereopure GalNAc-siRNAs appear to be driven by improved Ago2 loading. Ultimately, we generated HSD-1930, a stereopure GalNAc-siRNA targeting human *HSD17B13* based on our learnings from optimization of GalNAc-siRNAs targeting *Ttr*. In mice that express an *HSD17B13* human transgene, a single 3 mg/kg subcutaneous dose of HSD-1930 decreased expression of *HSD17B13* mRNA in the mouse liver by ≥75% of PBS-treated controls for at least 14 weeks post-dose. By comparison, *HSD17B13* mRNA levels in the liver of mice treated with a stereorandom control were comparable to PBS-treated controls at 14 weeks. HSD-1930's silencing advantage in mice corresponded with a significant improvement in Ago2 loading. Taken together, these preclinical data provide compelling evidence that HSD-1930 is a potent and durable silencer of hepatic *HSD17B13* expression.

1666 CD33 CAR T Cells Generated by Restrained TCR Stimulation and Optimized Cytokines Manifested Potent Antileukemic Activity and Less-Differentiated Memory Phenotype

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Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults with a low 5-year survival rate. Patients with unfavorable AML can't achieve long-term remission without allogeneic hematopoietic-cell-transplantation (allo-HCT), but the common subsequent relapse after allo-HCT often led to only a few months of overall survival. CD33 is a high-density antigen expressed on most AML blasts and is a safe immunotherapeutic target due to low expression on normal hematopoietic stem cells. We developed a CD33 chimeric antigen receptor (CAR) T cell product aiming to fulfill this unmet need for treating AML. Our previous clinical trial of CD123 CAR T cells was promising but identified the need for a faster CAR T cell manufacturing process. T cell proliferation and memory phenotype differentiation are heavily influenced by the strength of T cell receptor (TCR) signaling and γ-chain cytokines. We aim to shorten the manufacturing time of our CD33 CAR T cells without compromising product quantity and quality by optimizing αCD3/CD28 stimulation and cytokine combination. **Methods:** We cultured CD4+/CD8+ T cells isolated from AML patients (patient-derived; PD) or healthy donors (HD) with αCD3/CD28 Dynabeads at 1:3, 2:1, or 3:1 bead-to-cell ratio on day 0 and transduced with the lentiviral vector (huCD33scFv-CD8h-CD8tm3-41BB-Zeta(CO)-T2A-EGFRt_epHIV7) on day 1. We expanded the resulting CD33 CAR T cells in either IL2 (50U/ml) + IL15 (0.5ng/ml) or IL7 (10ng/ml) + IL15 (5ng/ml). We removed beads on either day 4 or 7 and evaluated cell expansion and immunophenotype by flow cytometry. We tested CD33 CAR T cell effort function in an AML cell model (Molm14 and KG1A) and in an AML xenograft NSG mouse model. We engrafted $1x10^6$ ffluc+Molm14 AML cells on day 0 and treated with $1x10^6$ PD CD33 CAR T cells on day 6, evaluated tumor growth via the bioluminescence imaging and monitored mouse survival. **Results:** We found that T cell expansion was higher, yielding more CD33 CAR T cells in the 4 day (4D) vs. 7 day (7D) condition. Lower bead ratio (4D/1:3-bead and 4D/2:1-bead) led to 8.6- and 11-fold more HD-derived and 2.1- and 1.4-fold more PD CAR T cells than the strongest stimulation group (7D/3:1-bead), respectively. The 4D stimulated PD CD33 CAR T cells were more effective against AML cell lines due to enhanced proliferation during tumor lysis assay: CAR T cells stimulated with 4D/1:3-bead and 4D/2:1-bead proliferated 9.0- and 3.1-fold more than the 7D/3:1-bead group, respectively. Regardless of cytokine condition, the 4D/2:1-bead condition consistently generated the most CD33 CAR T cells. PD CD33 CAR T cells showed similar expansion when cultured in either cytokine condition, while IL7/IL15 vs. IL2/IL15 led to 2.8-fold higher expansion for HD-derived CAR T cells. However, CD33 CAR T cells grown in either cytokine condition exerted comparable *in vitro* efficacy. *In vivo*, PD CAR T cells generated by 4D/2:1-bead with either cytokine condition dramatically limited tumor growth, but the condition of 4D/2:1-bead with IL2/IL15 resulted in the best survival

(100%) compared to 4D/2:1-bead with IL7/IL15 (33.3%) at day 71. Thus, we adopted the 4D/2:1-bead with IL2/IL15 for our clinical manufacturing platform. Production of CD33 CAR T cells achieved an average of 22.3- and 31-fold expansion on day 7 and 11, respectively, and manifested with a continuously increased proportion of Tn/scm (CD45RA+CD62L+) cells and decreased proportion of Tem (CD62L-) cells. **Conclusion:** Using a combination of restrained TCR stimulation (4D-2:1 bead) and IL2/IL15 cytokines, we optimized our CD33 CAR T cell production platform to yield highly proliferative CAR T cells with less-differentiated memory phenotype and potent antigen-dependent antileukemic efficacy.

1667 Development of Novel Size Exclusion Filters for Gene Therapy Applications

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Biotherapeutics are typically produced in mammalian cell lines which carries risk of viral contamination in the final product. Nanofiltration is one of the orthogonal steps used in biotherapeutics production to remove viral contaminants. There is a need for an easy-to-use nanofiltration system to remove large viral contaminants in downstream processing of biotherapeutics prior to formulation. In this study, 3M nanofilters were tested for their ability to separate small virus particles using bacteriophages PhiX174, QBeta and PP7 (25 to 28 nm) from larger bacteriophages Phi29 (45 nm) and T7 (50 nm) representing large viral contaminants. In addition, mammalian viruses ranging in various sizes were also tested. Processing was done at either constant flow (600 LMH) or constant pressure (30 psi). 3M nanofilters showed >6 log reduction of Phi29 and T7 and no retention of the smaller viruses. Similarly, 3M nanofilters showed >4 to 7 log of mammalian viruses (>35 nm). Additionally, process interruptions had no effect on viral clearance. The phage clearance studies show the potential of using 3M nanofilters as a platform for viral clearance studies for biotherapeutic applications.

1668 Oncolytic Adenovirus Armed with Multiple Immune Stimulatory Transgenes in Combination with PD-L1-Targeted Radioimmunotherapy Exert Synergistic Antitumor Effect Against Pancreatic Cancer

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Despite the remarkable advancements made in the immuno-oncology field, no immunotherapy regimen has been approved by US FDA for the treatment of pancreatic cancers to date. In detail, highly desmoplastic and immune-desert phenotype of pancreatic cancer are two major hurdles to the development of effective immunotherapy regimen, as these attributes markedly attenuate the capacity of cancer therapeutics and immune cells to penetrate and disperse throughout the tumor tissues to elicit robust antitumor immune response. To overcome these hurdles and develop an effective immunotherapy regimen against pancreatic cancer, a novel hypoxia-responsive oncolytic Ad (HY-oAd) co-expressing single-chain interleukin-12 (scIL12), granulocyte macrophage colony stimulating factor (GMCSF), and relaxin (RLX) and PD-L1-targeted immune checkpoint inhibitor (ICI), atezolizumab, radiolabeled with lutetium-177 (Lu-177; aPD-L1-Lu) were developed and their combined therapeutic efficacy was evaluated in desmoplastic and poorly immunogenic Pan02 syngeneic pancreatic tumor model. HY-oAd induced all three therapeutic genes in a dose-dependent and hypoxia-responsive manner in both human and murine pancreatic cancer cell lines. Importantly, HY-oAd treatment was shown to elevate PD-L1 expression level and promoted degradation of extracellular matrix (ECM) of pancreatic tumors, which led to elevated aPD-L1-Lu uptake. HY-oAd and aPD-L1 ICI therapy elicited more potent antitumor effect against the primary tumors than respective monotherapy in subcutaneous pancreatic tumor model due to enhanced infiltration and activation of $CD8^+$ T cells and significant attenuation of immunosuppressive regulatory T cells (Tregs). In an orthotopic pancreatic tumor model, HY-oAd and aPD-L1 ICI combination therapy showed superior antitumor efficacy against both the primary and metastatic tumors compared with monotherapy regimens. Importantly, the therapeutic effect of HY-oAd and aPD-L1-Lu was further augmented by addition of locoregional radiotherapy, which led to superior tumor growth inhibition and survival rate compared to HY-oAd and aPD-L1-Lu without radiation in both subcutaneous and orthotopic pancreatic tumor models. Collectively, our findings demonstrate that HY-oAd can enhance intratumoral accumulation of aPD-L1-Lu in a multifaceted manner to elicit synergistic antitumor immune response against desmoplastic and poorly immunogenic pancreatic tumors.

1669 Immune Synapse Optimized GRP78-Specific CAR T Cells Elicit an Improved Antitumor Response Against DIPGs

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Diffuse intrinsic pontine gliomas (DIPGs) are highly lethal pediatric brain tumors and a leading cause of cancer-related deaths in children. There is currently no cure for DIPG, highlighting an urgent need for novel therapeutics. Chimeric antigen receptor (CAR)-engineered T-cell therapy offers great promise for DIPG treatment; however, several limitations need to be addressed. In this study, we targeted a novel tumor associated antigen GRP78 because we showed it is reliably expressed on DIPGs. To test the anti-DIPG activity of GRP78-CAR T cells, we evaluated cytotoxicity, persistence, and cytokine secretion *in vitro*. We found that GRP78-CAR T cells kill DIPGs but fail to proliferate and secrete cytokines compared to positive control, U87. *In vivo* studies further confirmed that GRP78-CAR T cells eradicated U87 tumors but not DIPGs. Based on these findings, we concluded that DIPGs cannot trigger efficient CAR T cell signaling. Because CAR T

cell effector function is influenced by antigen density, we quantified the relative GRP78 expression on DIPGs and other brain tumors, including U87. We found that GRP78 is comparatively lowly expressed on DIPGs. Live-cell imaging of calcium flux in CAR T cells further revealed that T cell activation is lower upon interaction with DIPGs than U87s, suggesting that CAR-T cells form incomplete or dysfunctional immune synapses (IS) against DIPGs. To improve IS function, we knocked-out RASA2, a TCR downstream signaling inhibitor. Our results show that T cells that lack RASA2 have an improved IS reflected by bigger synaptic areas, increased lytic granules, and pZAP70 accumulation at the synapse. RASA2 KO further increased calcium influx and proliferation capacity of GRP78-CAR T cells against DIPGs. Thus, this study confirms that antigen density controls CAR T cell antitumor activity and improving the IS formation of CAR T cells elicits enhanced antitumor response against low antigen-expressing tumor cells.

1670 A Quantitative Method for Evaluating AAV-Mediated FKRP Expression and Function in Differentiated Myotubes

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Certain mutations in the fukutin-related protein (FKRP) gene prevent correct glycosylation of the structural protein α-dystroglycan (a-DG), leading to Limb-girdle Muscular Dystrophy 2I (LGMD2I/R9). Western blot and immunohistochemistry (IHC) analysis of muscle biopsies, whether for the detection of vector delivered WT FKRP protein or glycosylated a-DG using a monoclonal IIH6 antibody, have reliably confirmed expression of FKRP and correct glycosylation of its biological target in both research and diagnostic settings. However, development of robust in vitro assays which accurately measure both the expression of vector delivered FKRP and its downstream biomarkers remain an important objective in the field. There are technical challenges in the cultivation and transduction of differentiated myotubules, as well as the accurate quantification of glycosylated a-DG. Complete glycosylation of a-DG is not reached in cell culture models and a heterogeneous mix of different glycosylation states depending on stage of differentiation is often the result, which can obscure consistent quantitative measurements. We have developed a novel cell-based assay using immortalized myoblasts to assess the viability of adeno associated virus (AAV) vector gene delivery of FKRP. Important assay parameters were characterized to achieve maximum transgene expression/detection and target specificity. The downstream biological effect of FKRP expression, a-DG glycosylation, was quantitatively measured in a dose response using a plate-based ligand binding assay, which provides both high throughput and reliable quantitation of this downstream biomarker. This research may support the use of a single quantitative assay to characterize both transgene expression and its biologic function.

1671 Engineering Human Hematopoietic Stem and Progenitor Cells for Lineage-Specific Expression of Galactocerebrosidase Using Genome Editing

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Krabbe disease is a lysosomal storage disease caused by a deficiency in Galactocerebrosidase (GALCase) that affects the central and peripheral nervous system. Typically, symptoms are early onset and result in aggressive neurological deterioration with death in early childhood. The only available treatment is allogeneic hematopoietic stem cell transplant (allo-HSCT) which has significant morbidity and mortality, restricting its use to asymptomatic individuals with available donors. A potentially safer, more effective therapy for Krabbe disease might be achieved by engineering the patient's own hematopoietic system and consequently the monocyte/macrophage lineage to produce GALCase expressed at supra-physiological levels. This results in enhanced biochemical cross-correction, decreased morbidity, and eliminates the need for matched donors. Here we report an efficient CRISPR/Cas9 and AAV6-based approach that targets GALCase expression cassette with a monocyte/macrophage specific promoter to the CCR5 safe-harbor locus in human hematopoietic stem and progenitor cells (HSPCs). AAV donor repair templates were generated to drive GALCase expression with a lineage-specific promoter. This expression derived from CD68 promoter is restricted to the monocyte/macrophage lineage. This promoter was chosen to minimize potential complication of GALCase overexpression in the stem cell compartment. The allele targeting frequency of the CD68-GALCase vector was 29.75% and is sustained for 21-days in culture. The targeted cells generate about 1.5-fold increased galactocerebrosidase expression in macrophages and maintain multilineage differentiation potential in-vitro. By using a safe-harbor locus and lineage specific promoter to express GALCase, we minimize the risk of cytotoxicity in the stem cell compartment. Transplantation experiment into a immunocompromised mouse model will be required to determine GALCase HSPCs ability to maintain stemness and differentiation potential in-vivo. If further studies prove successful, the next step involves correcting Krabbe disease in a mouse model.

1672 Triaging Detergent Lysis for Downstream Purification of Recombinant Adeno-Associated Virus through Analytical Characterization, to Maximize Processing Yields

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The prevalence of AAV for use in gene therapies has brought about an increase demand for manufacturing. In so doing, the importance of

process improvement to lower manufacturing costs, improved process efficiencies, and increase production yields and purity is imperative. While there are many opportunities to reduce step losses at each phase of the manufacturing process none are more critical than at viral harvest from cells. The efficient release of virus from cells during recombinant vector production, if not done appropriately, can result in >50% loss in product due to inefficient lysis or precipitation/aggregation awaiting purification. To address this, we screened a panel of lysis reagents that are amenable for use in single step lysis of production cultures. As part of this panel, we included commercially available reagents, along with various detergents and salt combinations for a simplified single-step process and faster purification. Using our optimize purification methods of AKTA-based affinity chromatography, hydrophobic interaction chromatography and anion exchange chromatography, we demonstrate how lysis conditions directly impact viral recovery and analytical characterization to measure viral capsid titer, genome content, empty/ full ratio, capsid stoichiometry, host cell proteins and DNA content. This information should serve as a guide for improved downstream process controls the yield of recombinant AAV particles.

1673 Exploring Upstream Process Conditions to Improve Productivity of Novel AAV Capsids

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Recombinant adeno-associated virus (rAAV) has emerged as a promising technology for the delivery of gene therapies. There are multiple approved treatments and numerous (>100) ongoing clinical trials using wildtype serotypes. The rAAV field has focused on rare diseases because of the unmet medical needs of patients, the mechanism for correction (i.e., repair of a monogenic defect), and the amounts of material needed. As novel capsids with improved tissue tropism, better transduction efficiency, and reduced toxicity are developed, opportunities to treat larger patient populations and prevalent indications arise. To this end, scalable, higher-producing processes are needed to meet the material demands and, as the field matures, to help reduce costs. Suspension-based processes are more amenable to scale-up and are the basis for the work presented. Using HEK293 cells in suspension and a triple transfection process, bioreactor operating parameters and plasmid designs are tested. Bioreactors can support higher cell densities and allow greater parameter control compared to incubators and shaker flasks. Conditions are identified that improve titer and reduce residual impurity levels. The optimized bioreactor conditions are tested with multiple novel capsids to assess process robustness. Upstream changes potentially impact downstream steps. We look at harvest filter train sizing as part of assessing bioreactor changes. Collectively, the work here shows bioreactor conditions can be optimized to improve titer and process robustness for novel capsids identified from the platform.

1674 TAPE-seq is a Cell-Based Method for Predicting Genome-Wide Off-Target Effects of Prime Editor

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Prime editors (PEs) are powerful tools that widen the possibilities for sequence modifications during genome editing. Although methods based on the analysis of Cas9 nuclease or nickase activity have been used to predict genome-wide off-target activities of PEs, no tool that directly uses PEs for this purpose has been reported yet. In this study, we present a cell-based assay, named TAgmentation of Prime Editor sequencing (TAPE-seq), that provides genome-wide off-target candidates for PEs. TAPE-seq analyses are successfully performed using many different versions of PEs. The TAPE-seq predictions are compared with results from two other off-site prediction methods, Cas9 nuclease-based GUIDE-seq and Cas9 nickase-based Digenome-seq (nDigenome-seq). TAPE-seq shows a lower miss rate, and a higher area under the receiver operating characteristic curve compared to the other methods. TAPE-seq also identified valid off-target sites that were missed by the other methods.

1675 *TRAC*-Knock-In CD19CAR-T or gp350CAR-T Cells Targeting Burkitt Lymphoma with Type 1 or 2 EBV Infections Show Variable Potency, CD4/CD8 Ratios and Exhaustion Patterns

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INTRODUCTION: The ubiquitous Epstein-Barr virus (EBV) is an oncogenic herpes virus and is associated with several human malignancies. EBV is a notorious immune evasive pathogen that promotes $CD8^+$ T cell exhaustion and modulates $CD4^+$ T cell functions. Burkitt lymphoma (BL) is frequently associated with EBV latent infections, yet some cell lines show surface expression of the lytic antigen gp350. BL patients after relapse could profit from CAR-T cell therapy targeting cellular or EBV antigens. METHODS: We evaluated and compared the effects of CD19CAR.CD28z-T and gp350CAR.CD28z-T cells against $gp350^+$ $CD19^+$ BL. We used CRISPR/Cas9 methods to knock-in (KI) the CD19CAR.CD28z or gp350CAR.CD28z into the T cell receptor (TCR) alpha chain (*TRAC*) locus. RESULTS: For both CARs, the KI efficacy was roughly 20%. ^{KI}CAR-T cells were co-cultured *in vitro* with $gp350^+$ $CD19^+$ BL cell lines Daudi (type 1, generally latent EBV) or with Jiyoye (type 2, generally lytic EBV). Both types of CAR-T cells caused cytotoxic effects against BL lines *in vitro*, and the frequencies of $^{KI}CD8^+$ CAR-T cells at the end of the cultures were higher than $^{KI}CD4^+$ CAR-T cells. Co-culture of $CD19^{KI}$CAR-T cells with both BL lines showed upregulation of the activation / exhaustion markers PD-1, LAG-3 and TIM-3. For $gp350^{KI}$CAR-T cells, only Jiyoye

caused upregulation of the exhaustion markers. Two preclinical *in vivo* xenograft models were set up with NRG mice injected i.v. with $2x10^5$ Daudi/fLuc-GFP or Jiyoye/fLuc-GFP cells. Compared with the non-treated controls, mice challenged with either BL type and treated with $CD19^{KI}$CAR-T cells showed delayed lymphoma dissemination and lowered EBV DNA load. Administration of $gp350^{KI}$CAR-T cells into mice did not inhibit BL growth *in vivo*, but reduced the EBV DNA load of Jiyoye/GFPfLuc in bone marrow. Notably, $CD8^+$ $gp350^{KI}$CAR-T cells and $CD4^+$ $CD19^{KI}$CAR-T cells accumulated in the Jiyoye model. CONCLUSIONS: The type of CAR and the nature of the EBV^+ malignancy may contribute to different mechanisms of T cell development and therapeutic outcomes. Since CAR-T cells are being used clinically against lymphomas that carry latent or lytic EBV, it is important to take into account that EBV's immune escape mechanisms may interfere with the CAR-T cell properties and potency.

1676 Application of CRISPR Genome Editing to a Mouse Model of DFNA20/26

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Sensorineural hearing loss is a common disorder which affects the world's population. Previous inner ear gene therapy studies have shown promising results in improving auditory function in various mouse models of sensorineural hearing loss. While many studies focus on non-syndromic autosomal recessive hereditary hearing loss (DFNB), non-syndromic autosomal dominant hereditary hearing loss (DFNA) may be a better candidate for inner ear gene therapy translation due to the later onset and slower progression of hearing loss, which offers a wider therapeutic window for treatment. In this study, we applied CRISPR genome editing to a mouse model of human DFNA20/26. DFNA20/26 is caused by mutations in the *ACTG1* gene, which encodes gamma actin, an isoform of actin that is abundant in the inner ear. The $Actg1^{P264L/P264L}$ mutant mouse has an orthologous human P264L knock-in mutation and develops progressive hearing loss starting at ~P30 due to outer and inner hair cell stereocilia bundle abnormalities. In this study, guide RNAs (gRNAs) were designed and validated to target the P264L mutation *in vitro*. The gRNA with the best sensitivity and specificity for targeting the P264L mutation was delivered via adeno-associated virus (AAV) along with Cas9 nuclease into neonatal $Actg1^{P264L/P264L}$ inner ears via the posterior semicircular canal approach. We found that $Actg1^{P264L/P264L}$ mutant mice that were treated with CRISPR gene therapy showed improved outer hair cell stereocilia morphology compared to the untreated mutant mice. In addition, treated $Actg1^{P264L/P264L}$ mice showed improved ABR thresholds at ~P30 compared to untreated mutant mice. Our results demonstrated that CRISPR genome editing was able to improve the stereocilia morphology and auditory function in $Actg1^{P264L/P264L}$ mice.

1677 The Essential Role of K^+ and Ca^{2+} for Maintaining Cytotoxic Activity of Cryopreserved GAIA-102, a Newly Developed Activated NK-Like Cells

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Background: Previously, we established an original method to generate highly activated NK-like cells (GAIA-102) from mixed peripheral blood mononuclear cells from multiple donors. Now three phase I clinical trials, including neuroblastoma, for assessing the safety and potential anti-tumor activity are on-going. Before moving clinical trials, we and others demonstrated that cryopreservation of NK-based therapeutic modalities, including GAIA-102, impaired their viability and cytotoxicity. To solve this critical issue for off-the-shelf practice, we discovered that Plasma-Lyte A (PLA; a clinically available drip infusion) is a good candidate for maintaining viability and cytotoxicity of GAIA-102. We speculated that the substances contained in PLA have a protective effect on cryopreserved GAIA-102, and that other substances may have the similar function, even better. The aim of this study is to identify the substances which provide the protective effects on cryopreserved GAIA-102 cells.**Methods:** After generating GAIA-102, they were cryopreserved using STEM-CELLBANKER® EX and stored at -80℃ for more than 2 days, then used for following experiments. Immediately after thawing, the GAIA-102 were incubated with the test drip infusions, then, the cell viability and cytotoxicity were assessed. KCl, $MgCl_2$, $CH_3COO{\cdot}Na$ or $C_6H_{11}O_7{\cdot}Na$ was tested. Bumetanide (Na-K-Cl cotransporter inhibitor), digitoxin (Na^+-K^+ pump inhibitor) or verapamil (Na^+-K^+ pump and calcium channel inhibitor) was added to PLA.**Results:** At first, various clinically available drip infusions have been tested, and found that incubation of GAIA-102 with the drip infusions containing Ca^{2+} or glucose resulted in lower viability compared to that with PLA, while Ca^{2+}-/glucose-free drip infusions did not. The cytotoxic activity of GAIA-102 was decreased by depletion of K^+ or acetate from PLA, and it was increased by addition of these to saline. However, the viability was not affected by depletion of any substances tested from PLA. In these experiments, the greater effect on viability and cytotoxicity was observed in Ca^{2+} and K^+ containing drip infusions, respectively, therefore, we subsequently focused on these ions. Supplementation of K^+ to saline increased cytotoxicity at 1 mEq/L but subsequent addition up to 50 mEq/L did not induce further increase without any effect on viability of GAIA-102. When 0-32 mEq/L Ca^{2+} was added to PLA, viability was getting lower in Ca^{2+} concentration-dependent manner, however, cytotoxic activity was not, suggesting that incubation with Ca^{2+}-containing drip infusions would increase the cytotoxic activity of each GAIA-102 cell. To evaluate the effect of K^+ and Ca^{2+} uptake, GAIA-102 were incubated with PLA supplemented with K^+ or Ca^{2+} channel inhibitors, demonstrated that any inhibitors tested did not affect cell viability. On the other hand, verapamil decreased cytotoxicity in a concentration-dependent manner, while bumetanide

and digitoxin did not.**Conclusion**: Based on these results, we concluded that Ca^{2+} and K^{+} have an essential role for maintaining the cytotoxic activity of cryopreserved GAIA-102.

1678 Treatment of Infantile-Onset Pompe Disease in a Rat Model with Muscle-Directed AAV Gene Therapy

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Pompe disease (PD) is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme acid α-glucosidase (GAA), which leads to progressive glycogen accumulation, resulting in severe myopathy with progressive muscle weakness. In the Infantile-Onset PD (IOPD), death generally occurs early in life due to a cardiorespiratory failure. There is no cure for PD. Here, using CRISPR/Cas9 technology, we have generated the first rat model recapitulating key features of human IOPD. This rat model was used to assess a muscle-directed gene therapy based on the highly myotropic adeno-associated viral vector capsid AAVMYO3 and an optimized muscle-specific promoter, leading to widespread Gaa expression only in skeletal and cardiac muscles. Treatment of young PD rats with AAVMYO3-Gaa vectors mediated long-term correction of glycogen storage, prevented severe skeletal and cardiac muscle pathology, increased grip strength and rescued survival. This study provides a rationale for future clinical translation of this approach to treat PD.

1679 Rapid and Agile AAV Production Technologies: Cell-Free DNA Synthesis from Minicircles and Novel Bioreactor for Continuous Secreted AAV Collection

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Transient DNA transfection is commonly used to produce AAV vectors. cGMP plasmid production carries a high production cost and time burden (months to produce) due to: 1) complex bacterial-based manufacturing processes including establishment of master and working cell banks, large-scale bacterial fermentation, purification to remove immunogenic and toxic bacterial components, and related quality control assays, and 2) backlogs at the relatively few certified cGMP plasmid production facilities. This in turn can delay the advancement of gene therapy clinical trials. Thus, alternative methods that reduce cost, time and complexity are urgently needed, particularly for single patient production of AAV therapies for rare diseases. To address this issue, our group has been developing a cell-free synthesis approach to rapidly prepare DNA for cell transfection during AAV production. Our method produces gram quantities of rolling circle amplified (RCA) DNA from nanograms of circular templates in under one day. Cell-free production of RCA DNA uses defined buffer components, eliminating the need for host cell protein and endotoxin removal steps, and associated quality control assays. We have demonstrated that unprocessed, high molecular weight tandemly repeated RCA products can be used directly for transient transfection with high efficiency. We quantified AAV titers produced by triple-transfection of RCA DNA or plasmid DNA (encoding vector, rep/cap and adenoviral helper constructs) into suspension HEK293T cells, at up to 6 million cells per ml and up to 400 ml transfection volumes. Our data demonstrate that RCA DNA transfection produces ssAAV and scAAV with similar physical and biological titers as plasmid transfection. RCA DNA synthesized from minicircle DNA templates encoding the vector but lacking plasmid maintenance elements (e.g., antibiotic resistance genes) minimizes the risk of horizontal gene transfer of plasmid backbone elements into patients. We found that scAAV and ssAAV produced from plasmid, or from RCA DNA synthesized from plasmid templates, had a plasmid backbone sequence mispackaging frequency of ~20%. In contrast, RCA DNA synthesized from minicircle templates encoding the vector sequence had >10-fold lower mispackaging frequency. In parallel, we are developing a novel prototype single-use static perfusion bioreactor (SPB) for continuous secreted AAV collection from high density producer cells. The 300 cm^2 SPB supports cell densities up to ~$20x10^6$ cells/cm^2. Our initial evaluations tested the 300 cm^2 SPB using suspension HEK293T cells seeded at 3-$5x10^6$ per cm^2. The SPB enabled recovery of secreted AAV for 7 or more days post-transfection, with raw titers of ~10^{10} vg/ml. At the conclusion of a 7-day secreted AAV collection, the day 10 cell lysate still provided ~10^{13} vg. We are continuing to refine collection conditions. AAV titers from the SPB were comparable to titers obtained using the Xuri™ Cell Expansion System W25 (wave motion bioreactor). The raw secreted AAV collections were concentrated and purified using AAV-X affinity capture resins, providing ~90-100% recovery with a ~1000-fold reduction of total host cell protein content. In conclusion, the combination of these novel technologies may benefit personalized and agile AAV vector production for rare diseases for one to a few patients at a time.

1680 Zinc Finger Repressors Against Immune Checkpoint Molecules to Improve Anti-Tumor Activity of Gene-Modified T Cells

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The adoptive transfer of gene-modified T cells has emerged as a revolutionary new pillar in cancer therapy owing to the unprecedented clinical results obtained with chimeric antigen receptor (CAR) T cells in treating certain subsets of B cell leukemia and lymphoma. Despite this remarkable success, many barriers still limit a more broadened therapeutic efficacy in solid tumors and other hematological malignancies. These include severe toxicities, antigen escape, restricted trafficking, and limited tumor infiltration. In addition, T cell exhaustion and an immunosuppressive tumor microenvironment (TME) represent critical constraints to maintain effector functions and persistence and achieve a durable clinical potency. To overcome these significant challenges, innovative strategies and approaches are necessary to engineer more potent gene-modified T cells with improved anti-tumor activity. We recently described the generation of highly specific Zinc Finger Repressors (ZF-Rs) capable of silencing gene expression in T cells without the need for double-strand breaks. Their degree of gene repression is tunable, ranging from a partial knockdown to total gene silencing. Furthermore, their compact size allows the multiplexing of ZF-R sequences in a single lentiviral vector (LV) to achieve concomitant multi-gene silencing upon a single transduction event. Using this technology, primary human T cells were engineered with a bidirectional LV construct encoding one or two ZF-Rs, either with or without an anti-CD19-CAR, to knock down cell surface proteins described as negative regulators of the anti-tumor activity. These include immune checkpoints, successfully targeted by immunotherapy, and also factors playing a key role in the TME and the TCR signaling. We demonstrated that our ZF-R-expressing LVs could efficiently transduce both PBMC-derived CD3+ cells and tumor-infiltrating lymphocytes isolated from colorectal cancer patients. ZF-Rs repressed the target genes with high specificity at the mRNA level, resulting in efficient protein repression and this gene silencing was highly stable over time. Significantly, expression of ZF-R proteins did not affect *in vitro* functionality of gene-modified T cells, warranting further evaluation of their anti-tumor activity in animal models. Overall, our study proposes an innovative cell engineering platform to deliver gene-modified T cells with an improved tumor-eradicating potential.

1681 Assessing Gene Therapy Application in a Mouse Model of PTEN Hamartoma Tumour Syndrome

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PTEN Hamartoma Tumour Syndrome (PHTS) is an autosomal dominant disorder that is caused by germline mutations in the *PTEN* gene. PHTS is characterized by the development of multiple hamartomas as well as neurodevelopmental disabilities and autism spectrum disorder (ASD). The main function of PTEN is to regulate the PI3K/ AKT/mTOR signalling pathway which controls cell growth, proliferation, and survival. In the absence of effective treatment, continuous lifelong cancer surveillance and screening are critical for PHTS patients to allow early tumour discovery and treatment. PHTS is considered a good candidate for gene therapy due to the monogenic nature of the disorder and the lack of existing effective treatment. In addition, the *PTEN* coding can be readily packaged in AAV and the gene product may be secreted which could increase efficacy through cross-correction. Mouse models of PTEN haploinsufficiency show subtle neurological phenotypes but recapitulate other features associated with PTEN deficiency such as the development of multiple tumours, macrocephaly, and liver steatosis. In this study, we assessed the potential for gene therapy in preventing or ameliorating PHTS-like phenotypes in mice modelling *PTEN* haploinsufficiency. A codon optimized human PTEN transgene cassette was packaged in AAV9 and was injected systemically by tail vein injection into 5 weeks old $Pten^{+/\Delta 5}$ heterozygous female mice. A moderate dose (2.5×10^{13} vg/kg), and high dose (1×10^{14} vg/kg) groups were enrolled into the trial as well as cohorts of wild type and $Pten^{+/\Delta 5}$ mice were injected with vehicle (PBS) as controls. Experimental mice were monitored in a blinded fashion for the development and progression of gross/palpable tumours for 9 months. After mice were sacrificed, various tissues were collected for histopathological analysis. Our results show that 100% of $Pten^{+/\Delta 5}$ heterozygous mice, regardless of the treatment or the given dose, developed lymph node tumours with no treatment effect on the onset of tumour detection or tumour size. The lack of efficacy on tumour development could be explained by the very low virus copy number and the lack of detectable vector-derived PTEN expression in the lymph node tissue. In contrast, systemic injection of AAV9/*PTEN* increased PTEN level in the liver of vector-treated $Pten^{+/\Delta 5}$ heterozygous mice and significantly reduced the severity of liver steatosis (pre-cancerous lesion) and the monocytic infiltration observed in the vehicle-treated $Pten^{+/\Delta 5}$ mice. This study was limited by the fact that this mouse model develops gross tumours predominantly in the lymph nodes which are not efficiently targeted by AAV9. Development of a new capsid with higher lymph node tropism or crossing $Pten^{+/\Delta 5}$ mice with mice prone to hepatocellular carcinoma would be ideal to test the potential therapeutic effect of vector-derived PTEN on tumour development and progression.

1682 Safe and Effective Non-Viral Method for Delivering mRNA to the Lung Endothelium of Newborn Mice

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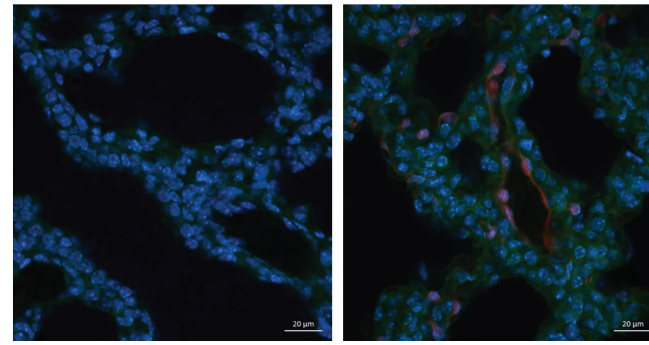
There have been rapid advances in the discovery and engineering of gene editing tools for monogenetic diseases in recent years. For the successful clinical translation of these therapies, there remains a need for safe and effective delivery vehicles. Poly(amine-co-ester) s (PACE) are cationic polymers with tunable chemistry that are capable of forming complexes with nucleic acids (i.e., polyplexes).[1] Various amine-containing groups can be conjugated to the end groups of the PACE chains to enhance endosomal escape.[2] PACE with an aminomethyl propanol end-group (denoted PACE-14) forms stable polyplexes that enable robust expression of mRNA in the lung epithelium after intratracheal administration.[3] We optimized PACE-14/mRNA polyplexes for intravenous (IV) use in the neonatal period as gene therapy at this developmental stage can prevent irreversible pathologies and access unique cell progenitor populations.[4] In this work, we demonstrate that the administration of PACE-14/mRNA polyplexes to newborn mice leads to mRNA expression in the lung endothelium without off-target expression in the liver or spleen. We formulated polyplexes encapsulating 0.25 μg of mRNA encoding for Cre recombinase and delivered the polyplexes via facial vein injection to Ai14 mice on postnatal day 1. Upon successful mRNA translation and subsequent Cre-mediated recombination, cells in the Ai14 mouse expressed robust TdTomato fluorescence. At 72 hr post-injection, we measured fluorescence in the lung, liver, and spleen by flow cytometry. We observed no TdTomato signal in the livers or spleens. In the lungs, we found approximately 55% of CD31+ endothelial cells expressed TdTomato. There was no expression in CD45+ lung cells or EpCAM+ lung cells. We confirmed these results by confocal microscopy of fixed lung tissue from uninjected and injected neonatal mice (Figure 1). None of the mice in either group exhibited gross signs of toxicity with the injection of the polyplexes. The use of PACE-14 polyplexes represents a safe and effective strategy for delivering mRNA to the lung endothelium of newborn mice. We hypothesize that IV administration of these engineered polyplexes will offer targeted delivery of gene-editing tools, such as Cas9 mRNA and sgRNA, to treat diseases of the pulmonary vasculature. One such disease is alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV).[5] This congenital disorder leads to pulmonary hypertension after birth and early death and is associated with mutations in the Forkhead Box F1 (FOXF1) gene.[6] We believe the PACE-14 technology can be used to deliver gene-editing tools to correct the associated mutations in ACDMPV and prevent pulmonary hypertension in these neonates.

Figure 1. Images of neonatal lung without injection of mRNA (left) and with intravenous injection of PACE-14/Cre mRNA polyplexes (right)



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1683 Mobilization-Based Chemotherapy-Free Conditioning for Hematopoietic Stem and Progenitor Cells Gene Therapy in the Non-Human Primate Model

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In conventional hematopoietic stem and progenitor cell (HSPC) gene therapy, the patient receives *ex vivo* genetically corrected HSPCs after conditioning regimens designed to reduce the number of resident cells in the bone marrow (BM) and free space for the engraftment of corrected ones. However, the chemo/radiotherapy-based conditioning procedures currently used in clinics may have severe acute and long-term side effects. To overcome this limitation and advance toward a chemotherapy-free non-genotoxic conditioning regimen, we previously reported that HSPC mobilization with G-CSF, AMD3100, and BIO5192, efficiently depletes the BM niche, creating a window of opportunity for the engraftment of *ex vivo*-cultured cells in a human hematochimeric mouse model. Additionally, transient overexpression of the CXCR4 homing receptor using an improved mRNA platform, showed the compatibility with gene transfer and CRISPR/Cas9-based homology mediated gene editing, conferring to the transplanted cells a competitive engraftment advantage over the mobilized recipient ones. Here, we explored the application of CXCR4 variants resistant to their antagonist AMD3100 used for mobilization to further increase the advantage of donor cells. mRNA-based transient overexpression of these variants on the cell surface endowed human HSPCs with partial resistance to CXCR4 antagonists both in *in vitro* migration assays and *in vivo.* In particular, we show that transplantation in hematochimeric mice of HSPCs overexpressing the drug-resistant CXCR4 variant led to

enhanced exchange with the mobilized recipient cells when compared to overexpression of wild type CXCR4. Moreover, transient overexpression of other homing and retention receptors, as KIT, ITGA4, and the anti-phagocytosis signal CD47, are currently under investigation as they may provide a synergistic effect when coupled with CXCR4. However, the hematochimeric mouse model may not fully reproduce the human HSPC-niche interaction and testing and validation of our exchange strategy in non-human primates (NHP) is required before planning its clinical translation. Our current results in this model are consistent with observations in human cells, showing that CXCR4 expression is rescued during *ex vivo* culture of NHP HSPC harvested by G-CSF and AMD3100 mediated mobilization and transient overexpression of this molecule increases SDF-1 dependent migration efficiency in *in vitro* assays. Moreover, we show that base editing at the CD33 locus and CXCR4 overexpression can be combined without impacting the efficiency of each procedure, conferring edited cells a migration advantage. Successful translation in the NHP model of our strategy leveraging on HSPC mobilization and transient overexpression of drug-resistant CXCR4 may open the path toward a broader and safer clinical application of HSPC gene transfer and gene editing minimizing the toxicity associated with current conditioning procedures.

1684 Hybrid Dual scAAV Delivery of Transgenes to the Kidney

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Gene therapy for kidney disease remains a challenge primarily due to lack of gene delivery to the kidney. Cystinuria, the most common inherited kidney stone disorder, results from a deficiency of an amino acid transporter (rBAT) that reabsorbs cystine in proximal tubular cells. Cystinuria patients suffer from cystine stones, obstruction, and development of chronic kidney disease; current effective treatments are lacking for this lifelong disease. We have previously shown significant reductions in urinary cystine levels in murine models of type A cystinuria through plasmid delivery of transposable elements containing *Slc3a1*, which encodes rBAT. However, gene transfer was estimated to be 1-5% of proximal tubular epithelial cells within the injected kidney and therefore did not affect cystine stone formation. Recent innovations in viral vectors have allowed for improved renal transduction. With a novel adeno-associated virus, AAV.cc47, we have shown efficient viral delivery to the proximal tubule in mice. However, the proximal tubule-targeting AAV utilizes a reduced viral genomic packaging size due to self-complementary DNA. In order to design a gene therapy strategy for type A cystinuria using this AAV, the transgene packaging limitations of the AAV necessitate the splitting *Slc3a1* into two AAVs. Full length *Slc3a1* expression can be induced by recombination of the split *Slc3a1* transgene using hybrid homologous recombination and mRNA splicing within the host cell. We have validated this hybrid reconstitution strategy to express *Slc3a1* in *in vitro*, in human kidney organoids, and in *in vivo* models of type A cystinuria. Further characterization of the cystinuria phenotype is necessary to assess potency and long-term efficacy of the dual-AAV gene delivery of *Slc3a1* to type A cystinuria mice. Phenotypic correction of a kidney disease has remained a challenge in animal models, but our current efforts to optimize the delivery, integration, and stable expression of desired transgenes through AAV and transposon engineering provide hope for overcoming the barriers to kidney gene therapy.

1685 Simultaneous Delivery of Multiple AAV Vectors by Phenolic Adhesives

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Introduction Adhesive properties of polyphenolic molecules have been widely used in interdisciplinary fields such as biomedical and energy devices. When encountering biological macromolecules such as proteins, polyphenols exhibited rapid complexation with serum or cellular proteins controlling their binding affinities and activities. Adeno-Associated Virus (AAV)-based gene therapy products have been approved by US and EU FDAs. While AAV is safe for human, the DNA packaging is limited to ~4.7 kB (corresponding to ~1,600 amino acids) which is one of main limitations in AAV therapeutics. Thus, simultaneous delivery of AAVs to specific cells or tissues is utmost important to address the gene size limitation. In this study, we propose preparation of supramolecular AAV assembly by adhesive phenolic molecules for simultaneous delivery of multiple genes. **Methods** We chose one naturally exiting polyphenol and one non-natural polyphenol to prepare supramolecular AAV assembled particles. For supramolecular assembly, naturally existing polyphenol was mixed vigorously with AAVs at a volumetric ratio of 1:1 and 9:1 for non-natural polyphenol. Ultraviolet-visible (UV-Vis) spectroscopy was used to estimate the formation of supra-molecularly assembled AAVs. For further size analysis Atomic Force Microscopy (AFM) was used. To demonstrate the cellular uptake efficiency by supra-molecularly assembled AAV, HEK293T (Human Embryo Kidney) cell line was used. After 48-hour infection, fluorescent images were obtained using fluorescence microscopy and fluorescence-activated cell sorting (FACS) was conducted to quantify the values. **Results** The AAVs solutions assembled via natural and non-natural polyphenols showed a little turbidity. If the complex exhibited microscale aggregation, A600 was expected to increase because of light scattering. Furthermore, AFM imaging was conducted for size analysis of AAVs. The height of original AAV showed ~22 nm height which shows the similar value as many researchers reported (~ 25 nm). In contrast, the height of the particle was shown to be in the range of 100 - 250 nm for naturally existing polyphenol assembled AAVs. For non-natural phenolic assembly, a slight increased size distribution was observed. Our *in vitro* experiments revealed improved co-transduction efficiency of dual AAV vectors by 76-80 % compared with no assembled AAV treatments. **Conclusion** This study demonstrated that use of polyphenol molecules is a promising approach to assemble AAV particles. In principle, the gene number delivered by AAV vehicles is many, but in practice, co-delivery of two or three genes is reasonable gene numbers. Attempts to increase the number of genes remains further an on-going study.

1686 Novel Machine Learning Protocol for the Phenotypic Prediction of AAV Variants Library Production

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The last decades have seen the important development of informatics lead to a revolution in the study of complex biological processes. Applying artificial intelligence (AI) methods to existing biological datasets offers an innovative strategy to predict biological properties.[1] Machine learning and deep learning are evolving branches of computational algorithms that use mathematical and statistical approaches to emulate human intelligence, using the surrounding environment as a learning step. Adeno-associated virus (AAV)-based capsid libraries are growing popular as candidate selection tools for gene therapy vectors, underscored by recent advances in the study of the specific VR-VIII region.[2] However, available data on mutations outside this region remains severely unexplored. To optimize the biological properties of AAV and generate suitable data on these regions, AAV diversity was generated through shuffling of amino acid 353-601 of natural capsids. This shuffling of natural AAV allowed the creation of a specific library composed by 272 capsids, mutated in 43 different positions along the 249 bp region. A machine learning (ML) model was then built, based on the two following protocols: (i) Learning from the sequence, we generated numerical descriptors characterising the physico-chemical properties of the mutated residues. (ii) Based on the sequence, the AAV2 variants were partially reconstructed as 3D objects, and a ML algorithm was trained on the geometric data. The following figure summarises these protocols:

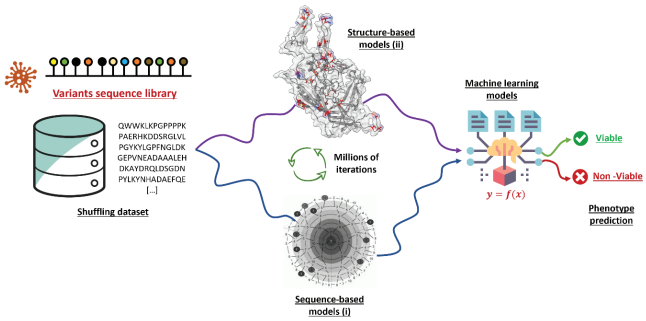

Figure: Schematic representation of the protocols used for the phenotypic prediction of AAV variants.

In total, more than a million models were generated through the variation of the tuning parameters, the cross-validation protocol, and different data partitions. Both models have different ways to predict the same output and achieve powerful performances: respectively (*i*) 100% accuracy, (*ii*) and 94% accuracy with associated specificity (66%) and sensibility (100%). In conclusion, experimentally produced shuffled AAV variants were successfully used to predict capsid viability for multiple mutations outside of the VR-VIII region. Our machine learning protocols result in powerful performances and lead to the identification of positions predicted as fundamental for the stability of the shuffled natural AAV capsid. As a perspective, additional experimental assessments will be performed on a selected list of variants mutated at the 43 positions identified as interesting by our AI protocols.

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1687 Peptide-Mediated Delivery of CRISPR Ribonucleoprotein (RNP) into Primary Human CD34+ Hematopoietic Stem Cells

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Following mobilization, cultured hematopoietic Stem and Progenitor Cells (HSPCs) can be edited by CRISPR-Cas9 *ex vivo* for cell therapy. Autologous transplantation of edited HSPCs has already demonstrated clinical success in addressing monogenic blood disorders such as β-hemoglobinopathies. Efficient and gentle intracellular delivery of the CRISPR ribonucleoprotein (RNP) complex is a remaining challenge, since the predominant technology is electroporation, which has substantial drawbacks including poor cell viability and dramatic impacts on cell state (which may, in turn, impair cells' ability to engraft). Inspired by viruses, which rely on peptide sequences with cell-penetrating capacities to cross cell membranes, we have designed and tested a library of amphiphilic peptides with cell penetrating and endosomolytic capacities. When combined with different CRISPR enzymes: Cas9 nuclease, adenine base editor (ABE8e), and Cas12a (Cpf1), our novel peptides can deliver CRISPR RNP to the nucleus of primary human CD34+ HSPCs and perform efficient genome editing (up to 82% indels via NGS) while showing only limited toxicity (≤20% dead cells). Our delivery approach produces therapeutically relevant levels of editing using either nuclease or base editor, surpassing the clinical threshold (>20%) for therapeutic benefit of sickle cell disease patients (up to 60% and 55% editing at the *BCL11A* enhancer via Cas9 and ABE8e, respectively). This approach maintains cell viability, proliferation, and phenotype as well as their potential to differentiate and perform erythropoiesis. Our delivery technology involves applying the RNP-peptide complex directly to cells in suspension by simply mixing them into the culture, with no complicated manipulation or hardware requirements (for either synthesis or administration of the RNP-peptide formulation). Importantly, all components of our system can be readily manufactured: recombinant protein and synthetic RNA & peptide. Finding a reliable, efficient, gentle, convenient, and cost-effective delivery method is crucial to improve and deploy HSPC-based cell therapies, especially since β-hemoglobinopathies are most prevalent in developing countries. Our CRISPR delivery system is an appealing alternative to existing methodologies, and may facilitate a semi-automated, point-of-care manufacture pipeline for precision cell therapies.

1688 The Asparaginyl Hydroxylase FIH1 Improves Oncolytic Virotherapy in GBM

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Glioblastoma (GBM) is the most common type of brain tumor in adults, and to this date remains a deadly disease with limited treatment options. Oncolytic viral therapy is emerging as a promising therapeutic strategy against notoriously resistant tumors, including GBM, for which there is now an oncolytic herpes simplex virus (oHSV) G47Δ, approved as a drug in Japan. The asparaginyl hydroxylase factor inhibiting HIF (FIH1) is located in chromosome 10q24, a region often deleted in GBM as an inhibitor of HIF1α-mediated transcription of VEGF-A, leading to decreased angiogenesis. We previously demonstrated targeting of FIH1 by a viral encoded miRNA in oHSV-infected GBM cells. In addition, our previous work shows that FIH1 negatively regulated oHSV-induced NOTCH signaling in GBM tumors. We have thus hypothesized that FIH1 reconstitution in tumors will block pro-tumorigenic NOTCH and HIF1α signaling. Using FIH1-overexpressing cells and a newly generated FIH1-expressing oHSV, we observed a reduction in vascular permeability, measured by intravital imaging, and decreased expression of angiogenesis markers. In vivo, combination of FIH1 overexpression with oHSV treatment resulted in enhanced survival in mice bearing intracranial GBM tumors. Furthermore, FIH1 overexpression correlated with the enrichment of genes associated with oxidative phosphorylation and ROS pathways, in addition to increased VEGF expression through a HIF1α-independent pathway, suggesting the potential for multi-modality therapeutic strategies.

1689 CAR-Tregs for Systemic Lupus Erythematosus

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Several common manifestations of Systemic Lupus Erythematosus (SLE) poorly respond to conventional medical treatments due to unstoppable immune-mediated tissue damage associated with altered architecture and function of the lymphoid organs. Hence the need for alternative strategies. A promising approach could focus on regulatory T lymphocytes (Tregs), which physiologically quench the autoimmune response and could promote tissue regeneration. Previous studies have shown that to be successful, Tregs must recognize relevant antigens, have limited off-target bystander effects and be safe. We describe here the results of a cell therapy strategy based on Tregs that overexpress FoxP3 and bear an anti-CD19 CAR (Fox19CAR-Tregs). They proved effective in vitro in restraining the activity of B cells, a key player in SLE, without unwanted pro-inflammatory action. In an ad hoc humanized mouse model of SLE, Fox19CAR-Tregs were extraordinarily effective, being able, despite a relatively short in vivo survival (14 days), to restrict autoantibody generation, to protect spleens, kidneys and lungs, to delay lymphopenia, a key feature of SLE, and to restore the composition of the human immune system in lymphoid organs, in absence of inflammatory effects or detectable toxicity.Therefore, a single infusion of Fox19CAR-Tregs is sufficient to break the vicious cycle that leads to autoimmunity and persistent tissue damage, allowing for the restoration of homeostasis. They thus represent an efficacious and safe strategy for SLE.

1691 Novel CRISPR-Associated Gene Editing Systems Enable Efficient and Specific TRAC/TRBC Knockout Triggering Robust Expression and Sensitivity of a T Cell Receptor Specific for Mutant KRAS G12D

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Adoptive T cell therapy (ACT) has demonstrated activity in solid tumors, but further optimization is needed to establish reproducibly effective treatments. T cells engineered with T cell receptors (TCRs) recognizing intracellular oncogenic drivers like mutant KRAS, the most frequently altered gene in human cancers, have the potential to induce durable responses in patients with solid tumors. TCRs are heterodimeric proteins consisting of a- and b-chains that can activate T cells following recognition of the desired target peptide presented by MHC. Competition between transgenic and endogenous TCRs for the available pool of CD3 proteins in T cells and the potential mispairing of transgenic and endogenous TCR chains can lead to decreased surface expression of the transgenic TCR, and thus diminished sensitivity. Here we employed programmable nucleases to genetically edit the constant regions of endogenous TCRs to eliminate endogenous TCR expression and mispairing with the transgenic TCR. We used a novel CRISPR-associated nuclease originally identified from environmental microbial samples and identified novel lead gRNAs that resulted in TCR knockout in >90% of human primary T cells. Since off-target activity is a potential safety risk, we characterized nuclease specificity using the 'gold standard' oligo capture assay followed by validation of any off-target activity using the Amplicon-sequencing assay. We demonstrated, with a sensitivity of detection approaching 0.1%, preclinical high specificity of editing, supporting safety of our gene edited TCR T cell therapy. To evaluate the functional impact of endogenous TCR

deletion, we edited primary T cells transduced with a lentivirus carrying CD8ab co-receptor and a TCR specific for the peptide derived from the KRAS G12D mutation presented in the context of HLA-A*11:01, one of the most common HLA alleles worldwide. TRAC/TRBC knockout improved expression of the transgenic TCR and improved the sensitivity of the engineered T cells, with TRAC/TRBC edited cells exhibiting enhanced *in vitro* cytotoxicity against tumor cells expressing the mutant KRAS G12D peptide. In summary, we describe the importance of genetic knockout of endogenous TRAC and TRBC for increasing the safety and functional potential of TCR-based cell therapy against solid tumors. The functional benefit and specificity of our genetic editing supports utility in clinical development of TCR-engineered T cell therapy for treating KRAS-mutant solid tumors.

1692 Focal Radiotherapy Improves the Efficacy of CAR T Cells Directed Against Prostate Cancer

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Chimeric antigen receptor (CAR) T cell therapy is an adoptive cell immunotherapy that has demonstrated great clinical efficacy treating hematological malignancies. Unfortunately, persistent and durable responses against solid tumors are limited due to low infiltration, trafficking, and activity of CAR T cells within the immunosuppressive tumor microenvironment (TME). These challenges can potentially be overcome by combining CAR T cells with other therapies that remodel the TME, alleviate immunosuppression, improve T cell infiltration, and promote endogenous immune responses. As such, our group recently found that pre-conditioning with lymphodepleting agent, cyclophosphamide (Cy), modulates the TME to become less hostile and enhances the recruitment and activity of endogenous and CAR T cells targeting the human prostate stem cell antigen (hPSCA) against mouse prostate cancer. Similarly, focal radiation therapy (RT) is also known to have immunomodulatory and tumoricidal effects that can alter the TME of otherwise immunologically "cold" tumors; however, little is known about how the immunomodulatory effects of focal RT may promote CAR T cells in the treatment of solid tumors. Our experiments have shown that combining Cy pre-conditioning with focal RT and PSCA-CAR T cell treatments significantly improves therapeutic responses and survival in mice engrafted with a single mouse prostate tumor. Furthermore, we empirically determined the optimal dose and fractions of RT that induced the most beneficial changes within the TME, including greater immune cell infiltration, dendritic cell maturation, and MHC Class I antigen presentation. To this end, we found that distal, non-irradiated tumors exhibited greater sensitivity to systemic PSCA-CAR T cells treatment following single tumor site focal RT treatment combined with Cy pre-conditioning. Cured mice who were rechallenged with wild-type antigen-negative tumors also demonstrated the longest delay in tumor out-growth compared to other initially cured mice treated without RT, indicating that the RT, Cy, and PSCA-CAR T cell treatments induced the strongest protective immune response. Our studies show that our focal RT combination treatment strategies enhance the anti-tumor activity of CAR T cells and may contribute to a more robust systemic immune response against metastatic burden in prostate cancer.

1693 Dasatinib: A Src Kinase Inhibitor to Prevent Anti-AAV Antibody Response and Enable AAV Re-Administration in Mice

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Introduction: Recombinant Adeno-associated virus (rAAV) is a promising vector for in vivo gene therapy due to its safety and efficacy and many rAAV-mediated gene therapy clinical trials are ongoing. Nevertheless the humoral response to the vector capsid remains a main challenge. Anti-AAV neutralizing antibodies completely prevent redosing and may be linked to some of the adverse events reported in clinical trials. For patients treated very early in life, readministration may be needed. It is therefore of high interest to investigate immunomodulatory strategies to prevent the humoral immune response against AAV vectors. We tested the effects of two different kinase inhibitors, dasatinib and acalabrutinib respectively targeting Src and BTK, on the antibody response to AAV capsid and efficacy of vector re-dosing in mice. **Methods:** Starting 2 hrs before the first rAAV dosing, 4 groups of 10 C57Bl6 mice were treated for one week only with dasatinib, acalabrutinib, dexamethasone or no treatment (control) respectively. Mice were immunized against AAV8 by intravenous injection of a rAAV8 coding for the human Secreted Alkaline Phosphatase (hSEAP) on day 0 and received a second intravenous injection of a rAAV8 encoding Factor IX on day 22. Both transgenes, hSEAP and hFactor IX concentrations were measured in the plasma over time until sacrifice on day 36. Anti-AAV IgM and IgG titers were also assessed over time, using ELISA assays. The effect of dasatinib was also investigated in vitro in human whole blood assays, to assess its effects on cytokine and chemokine release in response to rAAV. **Results and Conclusion:** Transient treatment with dasatinib led to a strong reduction in anti-AAV8 IgM and IgG antibodies and hence allowed transduction by a second rAAV8, leading to expression of the transgene product hFIX. Acalabrutinib treatment showed some but lower efficacy. Treatment with dexamethasone did not impact anti-AAV antibody formation. In some of the dasatinib- and acalabrutinib- treated mice, anti-AAV ADAs started to increase before the 2nd AAV8 injection suggesting that the treatment should be prolonged. AAV8 particles may persist in blood and tissues after the end of the immunosuppressive treatment. The immunosuppressive effects of the tested compounds were transient, as the treated mice had similar anti-AAV8 ADAs titers as untreated mice at day 36 showing that they had recovered immunocompetence. In the human whole blood assay system, dasatinib inhibited the AAV-dependent release of some cytokines in response to rAAV in vitro. Overall, transient treatment with the Src inhibitor dasatinib appears to be a promising strategy to prevent the humoral response to AAV8 and warrants further investigation in the context of AAV-mediated gene transfer.

1694 Patient-Derived iPSC-Neurons and Microglia for Modeling Friedreich's Ataxia Disease

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Friedreich ataxia (FA) is a neurogenerative disorder caused by homozygous GAA expansion mutation in the first intron of frataxin (*FXN*) gene. FXN is a mitochondrial protein, and its deficiency leads to mitochondrial iron overload, defective energy production, and oxidative stress. We previously showed complete rescue of neurologic, complications of FRDA in YG8R mice with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPC), and rescue was mediated by FXN transfer from engrafted HSPC-derived microglia to diseased neurons.. We next developed an autologous stem cell transplantation approach using CRISPR/Cas9-mediated excision of GAA repeats in FRDA patients' $CD34^+$ HSPCs that increased frataxin expression and improved mitochondrial functions. The aim of the current study is to understand FRDA neurodegeneration in the context of microglia and the complex interaction between HSPC-derived microglia and neurons in disease pathogenesis. To this end, we generated a unique cohort of four different FRDA patient-derived induced pluripotent stem cells (iPSCs) along with the CRISPR/Cas9-gene edited FRDA iPSCs, two familial carriers and two unrelated control lines. These iPSCs were then used for generating cortical neurons and microglia (iMG) and "mini-brains". FRDA-neurons displayed increased Caspase-3 expression and non-homogenous microtubule staining in neurites and cell body, characteristic of apoptosis, as opposed to edited-neurons and non-affected controls. FRDA-neurons also displayed increased mitochondrial fragmentation, partially degraded cristae structure, elevated superoxide production, and membrane potential loss, which were not seen in the edited neurons and controls. Similarly, FRDA-iMGs displayed activated, amoeboid morphology with compromised phagocytosis, and mitochondrial dysfunction while the edited-iMGs exhibit a phenotype comparable to controls. Utilising these two models, to understand the role of microglia in the neurodegeneration in FRDA, and its rescue by the gene corrected microglia.

1695 Intensified AAV Manufacturing Using a Scalable, High-Cell Density Platform Supporting Increased Specific Productivities

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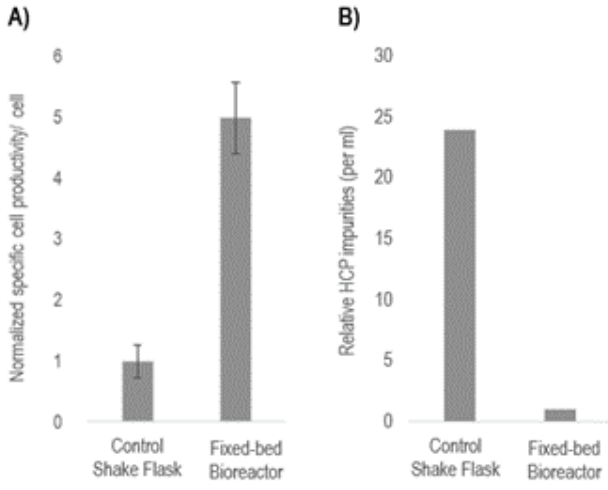
Considerable advances have been made in the monoclonal antibody industry to increase volumetric productivities, in large part through upstream intensification and the application of continuous processing. This has enabled manufacturers to generate large quantities of product using suspension-based processes in stirred-tank bioreactors (STR). While these improvements can provide a blueprint for commercial-scale manufacturing in the viral vector field, gene therapy developers remain far behind in replicating such successes. The inherent complexity of manufacturing processes, the unique characteristics of viral products, and scalability challenges are key factors in this struggle. The latter is especially pronounced for adeno-associated virus (AAV) developers aiming to achieve large-scale production while maintaining critical process parameters and meeting quality attributes. Limitations of STR systems, wherein key parameters such as shear, mixing and gas exchange cannot be simultaneously and linearly scaled-up, significantly constrain the design space for developers and can impact cost effectiveness of manufacturing processes.In this session, the speaker will present an intensified AAV manufacturing platform based on structured fixed-bed technology and designed to address the above-mentioned issues for both adherent and suspension platforms. By enabling transfection at >25x10^6 cells/mL without aggregation in a drastically reduced working volume, the upstream intensification of AAV manufacturing is unlocked, opening the way to cost-effective, large-scale production. The speaker will illustrate how a low shear, homogenous bioreactor environment leads to a 5-fold increase in specific productivity and present data supporting maintained performance across scales. The benefits of cell immobilization in the bioreactor will be described, circumventing the need for additional cell retention devices while achieving a >20-fold reduction in contaminating host cell protein. Finally, the speaker will present an integrated platform solution combining upstream and midstream operations, enabling combination of intensification with continuous processing for an optimized, low-footprint manufacturing workflow

Figure 1. Increased productivity and reduced impurity profile in a structured fixed-bed bioreactor. AAV production in suspension adapted cells in reference (shake flask process) compared to a structure fixed-bed bioreactor process. A) Normalized Specific productivity per cell. B) Normalized host cell protein (HCP) concentration per mL



1696 Stem Cells from Human Exfoliated Deciduous Teeth (SHED) Obtained by a Serum-Free Method Induced Significant Angiogenesis and Restored Blood Flow in a Rat Model of Critical Limb Ischemia

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Stem cells from human exfoliated deciduous teeth (SHED) are mesenchymal stem cells (MSCs) isolated from the dental pulp tissue. We developed a serum-free isolation/culture method and obtained the SHED with high proliferative capacity (KWB-SHED). KWB-SHED express surface MSC markers (positive for CD90, CD105 and CD73, negative for CD45, CD19, CD14, CD34 and HLA-DR), and have abilities of colony formation and trilineage differentiation same as conventional SHED cultured using fetal bovine serum (FBS-SHED). Microarray analysis revealed KWB-SHED highly expressed the genes associated with angiogenesis compared to FBS-SHED. Indeed, ELISA and multiplex analysis showed KWB-SHED secreted higher amount of angiogenic factors such as SCF, BDNF and ANGPTL4 than FBS-SHED on multiple donors. Furthermore, the conditioned media with KWB-SHED highly promoted proliferation of and tubule formation with human vascular endothelial cells (HUVEC) while the conditioned media with FBS-SHED had no effect. Next, we tested *in vivo* angiogenic activity of KWB-SHED using a rat model of critical limb ischemia (CLI). Intra-muscular administration of KWB-SHED to the ischemic limb showed a robust improvement of blood flow at the site of ischemia and avoidance of limb loss (Fig.1). Immunohistochemical analysis revealed a significant increase of blood vessels by SHED injection. Thus, KWB-SHED have high angiogenic capacity, suggesting potential clinical application for ischemic diseases requiring angiogenesis such as CLI.

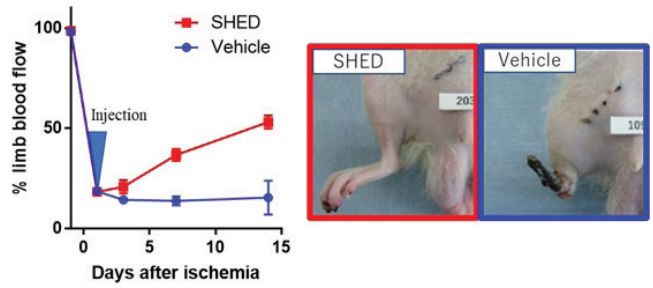

Fig. 1, CLI model result

1697 TALEN-Mediated Gene Editing of *LPA* Results in Gene Disruption and Plasma Lipoprotein(a) Reduction in Transgenic Mice

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Lipoprotein(a), or Lp(a), is an independent and causal genetic risk factor for cardiovascular disease. Individuals with high Lp(a) levels in circulation are up to 8 times more likely to suffer a life-altering cardiovascular event and are refractory to standard lipid-lowering agents. Lp(a) is a lipoprotein complex that contains apoB100 covalently bound to apo(a), the protein encoded by the *LPA* gene in humans. Apo(a) expression is restricted to the liver and Lp(a) levels in the circulation are genetically determined due to a repetitive exon that is polymorphic in human populations. Interestingly, natural homozygous loss of LPA in humans has not been associated with adverse phenotypes. These observations together suggest that LPA is an ideal candidate for a genome editing therapy geared towards reducing Lp(a) levels and resolving an unmet medical need. We designed TALEN®s, Transcription Activator-Like Effector Nucleases, that specifically target the human *LPA* gene and, after sequence optimization, the TALEN mRNAs were transfected into human primary hepatocyte cells to confirm their protein expression and gene editing activity. The *LPA*-targeted TALEN mRNAs were then formulated with Arcturus's proprietary LUNAR® lipid nanoparticle (LNP) and injected intravenously in a transgenic mouse model harboring a human LPA transgene. The LUNAR-LPA TALEN formulation targeted the liver and was well-tolerated. A single dose resulted in substantial reduction in plasma Lp(a) levels, which was sustained up to several weeks post-administration. Next-Gen amplicon sequencing and long-read HiFi sequencing confirmed the presence of gene-inactivating insertions and deletions at the *LPA* locus. *LPA* transcripts were reduced in liver tissue as evidenced by RNAseq and RT-qPCR, further confirming gene disruption. We also found that plasma levels of plasminogen, which is highly homologous to apo(a), were not affected suggesting minimal off-target gene editing. Overall, this study serves as a proof-of-concept for using TALEN-mediated gene editing to disrupt *LPA in vivo*, paving the way for the development of a long-term gene editing therapy for patients with high Lp(a).

1698 Fitness Maturation of Engineered AAV Capsids STAC-102 and STAC-103 Enhances Central Nervous System Transduction after CSF Administration in Cynomolgus Macaques

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The clinical translation of genomic medicines to treat disorders of the central nervous system (CNS) has been hampered by inefficient gene delivery. AAV administration into the cerebrospinal fluid (CSF) enables access to the CNS with relatively low doses and limited exposure to pre-existing anti-AAV antibodies. However, the level of CNS delivery mediated by conventional AAV serotypes is lower than is needed to affect many neurological diseases. We previously applied the functional selection platform SIFTER (Selecting In vivo for Transduction and Expression of RNA) to identify capsids with improved CNS transduction after CSF administration. The engineered capsids STAC-102 and STAC-103 exhibited up to a 100-fold enrichment in both vector genome biodistribution and mRNA expression compared to AAV9 across many CNS regions. Here we applied fitness maturation strategies to further enhance the performance of capsids STAC-102 and STAC-103. Focused fitness maturation libraries were constructed to

evaluate how combinations of capsid mutations influence potency and manufacturability. Transduction efficiency was assessed *in vivo* after intra cisterna magna delivery in cynomolgus macaques and *in vitro* in human iPSC-derived neurons and primary mouse cortical neurons. Capsid manufacturing yield was assessed in suspension HEK293 cells. Each capsid in the library was synthesized with three unique barcodes and each barcode was coupled to multiple unique molecular identifiers. Variants of STAC-102 and STAC-103 were ranked based on multiple factors including fold enrichment in mRNA expression, DNA vector genome delivery, and unique molecular identifier counts. Results from these experiments showed that minor changes to the inserted peptide or flanking capsid sequences can significantly impact both capsid potency and manufacturing yield. Moreover, we found a subset of mutations that were globally beneficial in the tested assays, while others showed substantially divergent outcomes. In cynomolgus macaques we identified capsid variants with improved CNS delivery relative to the first generation STAC-102 and STAC-103 capsids. We are evaluating these new capsid derivatives as potential candidates for therapeutic approaches to treat neurological disorders.